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SYNTHESIS AND EVALUATION OF SOME ARYLALKENYL AND ARYLEPOXYALKYL HYDROGEN SUCCINATES AND HYDROGEN GLUTARATES AS INHIBITORS OF RAT LIVER β-HYDROXY-β-METHYLGLUTARYL COENZYME A REDUCTASE

Paul Emil Marecki

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by

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With fond thoughts of Susan and Andrew....and an Omnilingual Flower for Algernon

Throughout the course of this work my professor, Dr. Marvin R. Boots, has provided much more than the physical necessities for research. Through the joy and pain of life he has always given freely of his patience, understanding, and knowledge while never requiring reward or repayment. It has been my privilege to work and learn with such an individual.

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INTRODUCTION - HISTORY AND BACKGROUND

A. Atherosclerosis: Pathogenesis and Etiology

The World Health Organization (1) defined atherosclerosis as "a variable combination of changes of the intima of arteries consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits and associated with medial changes." Thus, the major characteristic aspect of the disease is thickening of the arterial walls caused by deposition of various lipid substances. Both human and animal, and experimental atherosclerosis have been studied and described for many years (2, 3), however, only in the last twenty years have experimental techniques made possible detailed pathological and biochemical descriptions of the atherosclerotic lesion and its development.

The consequences of atherosclerosis may be extremely severe since it contributes to or aggravates several other diseased states. Atherosclerotic disease is an almost universal affliction and its occurrence and intensity increase with increasing age of the victim. The major results of the disease include possible bursting of the artery walls, proliferation of arterial tissues causing blockage of the artery or the induction of clotting which may interfere with or stop normal blood flow (4). Additionally, narrowing of the arterial lumen may cause an

ischemic reduction of blood flow to an organ or an occluding plaque may become dislodged and later become trapped in a vessel or artery causing myocardial or cerebral infarct (5). The time required to develop such a condition is measured in years or decades but the resulting accident which may lead to death takes place over a period of seconds. Often, death due to atherosclerosis is premature since at the time of death the unaffected bodily organs are usually functioning well and could have been expected to continue to do so for several more years (4). Therefore, a cure or at least an inhibition which would retard the progress of this disease would be expected to increase the life expectancy of the victim.

In the large elastic arteries atherosclerosis appears to begin as a focus where the innermost layers of the artery wall exhibit indications of damage followed by growth and repair of the tissue (4). The repair processes usually include cell proliferation and the production of new fibrous tissue. This very early lesion does not contain abnormal quantities of lipids.

Soon after the initiating event the first major type of lesion, the fatty streak, begins to develop. This is a flat intimal lesion which stains with the lipid stain Sudan IV and causes very little obstruction of blood flow (5). The predominant cell in the lesion is the modified smooth muscle cell, which is elongated, has a central nucleus, and contains longitudinally arranged myofilaments

in the cytoplasm (6). In human fatty streaks fat droplets begin to accumulate in these smooth muscle cells to such an extent that the cell becomes filled with lipid and is called a foam cell (4, 7). Most of the lipid enters through junctions between the endothelial cells or, perhaps, by pinocytosis (8). Esterified cholesterol is the major and triglycerides are the minor component of these lipids.

As the accumulation of fat in this lesion continues more foam cells appear. This process continues until some foam cells in the center of the lesion die, perhaps from anoxia. The intima and inner media of the aorta obtain oxygen by means of diffusion from the lumen (6). Any factor which causes interference with oxygen diffusion (such as proliferation of cells or accumulation of lipid) may inhibit respiration of the foam cells. It is therefore reasonable to suggest that continued lipid deposition may contribute to oxygen starvation of the cells in the developing lesion, resulting in death and necrosis of the cells. This is supported by the observation that hypoxia increases susceptibility to atherosclerosis while high oxygen tension appears to protect against the process (6).

The lipid released from dying foam cells begins development of fibrous plaques, the second major type of atherosclerotic lesion. This is a firm lesion which contains a fat base covered by a layer of fibrous connective tissue (5). Since this is a raised lesion it may cause some obstruction of blood flow. The lipid portion which

is released from foam cells contains amorphous cholesterol, some cholesterol crystals, triglycerides, phospholipids, and steroids (4). The cells around this deposit are irritated by the cellular lipid debris and respond by proliferation to form a thick, fibrous cover over the lesion. The extracellular fat may be phagocytized by macrophages, which may also develop into foam cells by the continued uptake of fat. If these cells do not or are not able to migrate away from the lesion they may disintegrate and contribute their contents to the lipid deposit. The fibrous plaque spreads down into the artery wall destroying the elastic lamella until the entire media has been transformed into a lipid paste. As this process continues cellular debris from destruction of arterial structural cells contributes to the growing plaque. This lesion is capable of growth which is so extensive that it may break through the outer adventitial vascular coat.

The final stage of plaque development consists of the appearance of insoluble calcium deposits (4, 5) which occur throughout the plaque and contribute to hardening of the lesion. This phase may be accompanied by vascularization by a system of capillaries, which may be an attempt to provide nutrition for any cells remaining alive.

At this stage of development the lesion has caused no major compromise of vessel functions. The destructive phase begins when a piece of the plaque becomes dislodged from the site of the lesion. The dislodged portion of

plaque may be carried away by the blood stream and become trapped in a narrow vessel. This contributes to occlusive damage. The gap remaining in the lesion after loss of a portion of plaque may become filled with blood and form a clot. Continuation of ulceration in this manner may lead to hemorrhage (4). The artery wall may become so weakened that it bulges outwards. The resulting aneurysm is a serious complication since it is capable of either pressing on an adjacent organ or rupturing. Rupture of the artery occurs most often if the vessel passes through soft or yielding tissue, such as the brain, and is enhanced by high blood pressure.

Another serious atherosclerotic complication is thrombosis, which appears to occur more often in atherosclerotic than in normal arteries and more often in individuals with high blood lipids and hypertension. Although the mechanism of thrombus formation related to atherosclerosis is not well known, microscopic cracks in the surface of plaques may initiate its development (4).

Study of atherosclerosis and its complications is hampered by the lack of a readily available, suitable experimental model for the disease. Atherosclerosis in man, the object of most concern with respect to the disease, can be studied only after death since the observation of arterial interiors cannot readily be repeated with the same subject. However, observation of man does provide important information concerning possible factors which

contribute to the disease. Problems associated with the choice of a suitable experimental animal are centered on differences in the disease process between the animal model and man. In most cases the conditions required for development of atherosclerosis in an experimental animal are too different from those apparently necessary for spontaneous development in man, or the lesion itself is considered too different from the lesion found in man (9). Perhaps the best available model is the nonhuman primate; some species of monkey develop atherosclerosis spontaneously with aging as well as by feeding lipid rich diets which are similar to the diets related to the disease in man (9). However, the necessary facilities for housing and caring for primates and the extended periods of lipid feeding required to develop the disease often make it impossible to use this animal as an experimental model. Perhaps because of the ease of inducing lesions and the reproducibility of these lesions, the rabbit has been the major experimental animal studied. Thus, much of the experimental data related to atherosclerosis in man has been derived from observation of rabbits as well as rats and may therefore be considered circumstantial evidence or proof by analogy. However, the overwhelming nature of this data provides some support for its fidelity.

There are several theories which attempt to explain the mechanism by which atherosclerotic lesions are initiated. The single unifying feature of the various theories

is the experimental fact that the lesion is composed of lipid material of uncertain origin. One of the theories with much support is the filtration theory. Very simply stated this theory explains that arterial lesions are derived from filtration of plasma components into the arterial wall (10-12). The initiating event for this infiltration of lipid is thought to be some irritation or injury to the arterial intima. Accumulating lipids and lipoproteins are irritants and they provoke a proliferation reaction by the arterial tissues (4, 11) which results in a fatty metamorphosis of the intimal cells, ending in disintegration. This is based upon the fact that most vessel walls do not have a vascular system, and therefore the metabolic needs of the wall are supplied by continuous diffusion of fluid from the lumen to adventitial lymphatic channels. When the blood lipids reach high levels, some of the largest lipids and lipoproteins are not able to pass completely through the artery wall and they begin to accumulate causing a proliferative response by the arterial wall. Support for this theory is derived from the observation that human populations with abnormally high concentrations of blood lipids exhibit a higher occurrence of atherosclerosis than populations with lower blood lipid concentrations. Further, high blood lipid levels in animals are known to induce atherosclerotic lesions similar to those found in man.

Recent support for the filtration theory is the work

of Colton et al. (13) who studied ultrafiltration of lipoproteins through synthetic membranes. These workers found that ultrafiltration of solutions containing low and very low density lipoproteins was accompanied by the formation of a lipoprotein layer on the membrane, a phenomenon known as concentration polarization. It was proposed that concentration polarization may be the major controlling factor for the movement of lipoproteins into arterial walls. Low density lipoprotein concentrations are important in coronary heart disease as well as atherosclerosis and these results suggest that low density lipoprotein transport into arterial walls would be favored by increased concentration polarization under conditions of high concentrations of low density lipoprotein relative to very low density lipoprotein.

Zilversmit (14), however, has shown that simple filtration may not be the sole governing factor. Study of rabbit aorta lesions induced by high cholesterol diets showed that the accumulated cholesterol in the lesions was derived from circulating blood. The kinetic studies of lipid influx and accumulation suggested that, rather than being governed by simple filtration and retention, there was a gradual increase of permeability of the artery wall which promotes influx and retention of serum lipoprotein and cholesterol. This evidence indicated that the filtration of lipid material into arterial walls was enhanced by permeability changes of the wall itself and provided

further support for the basic mechanism of filtration theory.

An interesting variation of lipid filtration theory has been suggested by the work of Constantinides (4, 12) who has emphasized the importance of injury to the arterial wall. These experiments showed that arterial walls which have been either chemically or physically injured exhibit faster lipid deposition at lower blood lipid levels than uninjured arteries. Furthermore, injury alone did not appear to lead to atherosclerotic lesions, but in the presence of elevated blood lipid levels lesion development was rapid. Agents capable of arterial injury include free fatty acids, desoxycholate, and phosphatidyl ethanolamine when infused directly into the arterial lumen.

The injury modification of the lipid filtration theory maintains that under normal conditions small molecules such as oxygen, water, electrolytes, sugars, and free fatty acids readily cross the endothelium and that large molecules do not (12). After arterial injury the endothelial junctions become so widened that the entire plasma with its lipoproteins may enter the wall and begin the lipid accumulation process. In this context injury includes necrosis and sloughing off of cells and may be caused by anoxia, amines, and high concentrations of lipids. Other investigators have also found evidence for the entrance of plasma into the arterial wall through endothelial junctions (15).

When injured artery wall is exposed to lipemia, atheromata may form either at the center or margins of the injury site (4). Atheromata developed from the center of an injury site are indistinguishable from early human fatty streaks. If these lesions are allowed to grow they develop into plaques which are also indistinguishable from those found in the human disease. Those plaques which develop from the marginal areas of injury sites grow into foam cells.

The thrombogenic theory differs considerably from the filtration theory in explaining the origin of atherosclerotic disease. The thrombogenic theory maintains that atherosclerotic plaques are initiated by flat or mural microscopic thrombi on the surface of arterial walls. It is postulated by this theory (11) that in the circulation fibrin is constantly formed and degraded by fibrinolysis. When the rate of fibrin formation exceeds that of fibrinolysis the excess fibrin becomes deposited on an artery wall and initiates formation of a thrombus. Initiation of the true plaque takes place when the thrombus is incorporated into the arterial wall by overgrowth of epithelial and vascular cells (4).

The most serious deficiency of the thrombogenic theory is its inability to account for the formation of fatty dots and streaks which are presumably the earliest gross expressions of the atherosclerotic process (11). The theory requires all early lesions to be in the form of

mural thrombi deposited on normal arterial wall tissue (12). Thrombi observed in human atherosclerotic aorta appear to take an intermediate position in development of the lesion since they are usually found on the atherosclerotic wall with thick fibrous caps or on thick fibrous arterial walls. Additionally, the thrombogenic theory does not provide for transitional forms between the early mural thrombus and the plaque stages of development. Thus, it appears that thrombi make a much more important contribution to growth and development of atherosclerotic lesions than to their initiation.

Each of the major theories concerning initiation and development of atherosclerotic lesions accounts for a vast body of experimental observation. However, each theory contains deficiencies in its explanation of experimental data. Similarly, each contains common principles or concepts which relate it to the others. Atherosclerosis and its complications provide a problem of such a complex nature that no single theory can be expected to account for all its aspects at this time. Therefore, rather than attempt to formulate comprehensive theories it is probably more productive to consider the process as the result of several factors acting in concert to produce the observed disease state. Several of these factors are apparently unrelated and require further study to clarify their respective contributions.

Several factors have been shown to be correlated with

the incidence of atherosclerosis and on this basis they are said to contribute to or predispose an individual to the disease more so than if a particular factor were absent. Many of these contributing or risk factors are involved with or are part of common daily practices. Because of interrelationships among some of these factors and the habitual nature of others they have often been collectively referred to as the effect of "life style" on disease development. Among the more important contributing factors are blood cholesterol and triglyceride levels (5, 16, 17), hypertension, diabetes and cigarette smoking (5, 17), and exercise (18, 19). Although they probably do not contribute to the disease directly, age and sex have been shown to be highly correlated with its occurrence; atherosclerosis is more prevalent in advanced age and is very rare in premenopausal women.

The incidence of atherosclerosis has risen very sharply since the beginning of the twentieth century. This is not an indication that the disease is new because evidence of it has been found in Egyptian mummies over three thousand years old. The increased observation of atherosclerotic events since the turn of the century is probably due to the use of chemotherapeutic agents (20). The prevalence of infectious diseases, especially tuberculosis, in the early years of this century caused death at an early age. Because serious complications associated with atherosclerosis do not occur until late in life the many

victims of death by infectious diseases did not live long enough to become susceptible to atherosclerotic conditions. The advent of chemotherapy removed infectious disease as a major cause of death and drastically increased life span allowing much larger segments of the population to live long enough to become victims of atherosclerotic diseases. In addition, more accurate documentation of the cause of death during this century has increased the awareness of atherosclerosis.

Thyroid function has been proposed to be an important factor in the development of atherosclerosis; thyroid deficiency enhances atherosclerosis and thyroid administration delays the disease in experimental animals (20). In an attempt to determine if this relationship is valid in humans, Barnes (20) studied 70,000 autopsies performed in Graz, Austria. This region of the world exhibited an extremely high incidence of goiter until iodized salt was introduced in 1963. The autopsies indicated a strong positive correlation between thyroid deficiency and occurrence of atherosclerosis. This suggested that premature death from coronary disease might be prevented by thyroid treatment. Thus, after administering thyroid therapy to 1569 patients for approximately six years only four new cases of coronary disease were observed, whereas 72 were expected from comparing the experimental group with a control group matched for age, sex, hypertension, and hypercholesterolemia (20). It should be noted that this

apparent 94% protection against atherosclerotic complication of coronary disease was associated only with thyroid therapy since there were no reported changes in diet, smoking, or exercise patterns.

Physical activity may play an important role in the development of atherosclerosis. In a study of Irish born brothers, one of whom lived in Ireland, the other in Boston, Brown et al. (18) were able to examine the effect of nutrition on atherosclerosis. The choice of brothers eliminated both hereditary and racial differences among the subjects and placed the emphasis of the study on nutrition and mode of living. With very few exceptions trends of food consumption in Ireland and Boston were very similar; although the Ireland brothers consumed 3700-4300 calories per day while the Boston brothers consumed only approximately 3000 calories per day. In light of the similar nutritional conditions in the two experimental groups, the U. S. death rate for atherosclerotic and myocardial disease was 300 more per one hundred thousand than that observed in Ireland (using 1950 statistics). Furthermore, the death rate increased the longer an Irish family had lived in this country. Since the Irish brothers consumed more calories but weighed less than their Boston brothers it was concluded that the Irish brothers were more physically active and stored fewer calories as fat. Thus, it was suggested that the quantity and general quality of food intake was less important when a large proportion of calories consumed

were expended by physical activity (18). This study indicated the importance of exercise in prevention of atherosclerotic development.

A possible physiological explanation of the apparent protection afforded by exercise is the observation of anoxia in the arterial wall. It has been observed (19) that the activity of certain enzymes in the human aorta decreases with advancing age and increasing severity of atherosclerosis, the loss being most severe in the middle media. This observation has led Adams et al. (19) to propose that the loss of enzyme activity may be due to anoxia. Since lipids enter the intima from the lumen and move outward, anoxia of enzyme systems responsible for transport or metabolism of these lipids may cause them to accumulate at the site of entry and therefore contribute to an atheromatous lipid deposit. It also seems reasonable that the reverse process could take place, that is, the accumulation of lipids by any of the previously discussed mechanisms could contribute to anoxia which, in turn, contributes to further lipid deposition. Thus, it is tempting to suggest that physical exercise may prevent anoxia of the affected enzyme systems and therefore make lipid accumulation an unfavorable process.

Smoking is also a risk factor for atherosclerosis (17) and may be related to anoxia. Decreased oxygen supply caused by vasoconstriction and decreased oxygen carrying capacity of the blood due to increased carbon monoxide

levels could contribute to the effects of anoxia.

Turbulence of blood flow has also been implicated in the genesis of atherosclerosis; however, the validity of this hypothesis is in doubt. The stenosis or arterial constriction used to study this effect is accompanied by increased blood pressure proximal to and turbulent blood flow behind the constriction. It is therefore uncertain if the relationships between arterial stenosis and atherosclerotic lesions are due to hypertension or to turbulent blood flow. Aars and Solberg (21) were able to cause a stenotic constriction of the middle ascending aorta of rabbits in such a manner that distal turbulence of the blood flow was produced without proximal hypertension. Development of atherosclerotic lesions in these animals by cholesterol feeding showed that the stenosis did not influence the location or extent of lesions. These data indicate the absence of a relationship between turbulent blood flow and the development of lesions, and indirectly support the view that hypertension is an important contributing factor.

Triglycerides are also frequently observed constituents of atherosclerotic plaques. An increase of the circulating triglyceride levels, even though cholesterol levels may be considered normal, is a condition predisposing to atherosclerosis (16). Both cholesterol and the triglycerides are important contributors to lesion development. For a given cholesterol level, coronary disease increases with

triglyceride concentration in the blood; similarly, for a given triglyceride level incidence of these diseases increases with cholesterol concentration (16). Therefore, high cholesterol and high triglyceride levels in the blood are both independent risk factors for atherosclerosis.

The most important contributing factor for atherosclerosis is the concentration of lipids, especially cholesterol, in the blood. Of all the lipids found in the blood and plaques, cholesterol is probably the most strongly implicated lipid in atherogenesis. A sixteen year follow-up study of the Framingham study (17) found that of the 5209 adults examined the serum cholesterol level was one of the highest correlating factors in development of atherosclerotic diseases. The high correlation between serum cholesterol levels and atherosclerosis is diagnostically valid only when comparing geographically or racially distinct populations and not when comparing individuals within those populations (5). This fact is illustrated by the wide variation in the extent of lesions in individuals from homogeneous subgroups.

Serum cholesterol levels have been correlated with the occurrence of atherosclerosis in a vast number of studies (22); however, the circumstantial nature of this correlation is often noted by the investigator. Similarly, a logical step in the treatment of a disease is removal of the causative factors. In the case of atherosclerosis, since there is no absolute proof that cholesterol causes

the disease, some investigators feel that therapy by removal of this tentative causative factor would only probably be beneficial but, at least, would not be harmful (23-25). This apparent confusion can be interpreted as another illustration of the complexity of atherosclerosis and that many factors acting together contribute to the development of the disease. Because of the large body of experimental evidence, cholesterol can indeed be considered to be intimately involved with atherosclerosis and its removal may very well prove to be beneficial.

The correlation between cholesterol and atherosclerosis is an old one; early investigators (26) used cholesterol feeding to study the pathogenesis of the disease in rabbits. Cholesterol is known to accumulate in human arteries and its deposition is a process which requires extended periods of time. The lipid composition of early lesions is very similar to that of blood plasma (24), suggesting that lipids found in these lesions are derived from the blood. As growth progresses the amount of cholesterol in the plaque increases until, in the late stages, it is the major constituent. This is further indirect evidence that lowering serum cholesterol levels should inhibit atherogenesis.

A smaller body of evidence exists which indicates no relationship between cholesterol and atherosclerosis. In an attempt to surmount the problem of not being able to examine the human aorta before death Landé and Sperry (27)

studied serum cholesterol levels and the severity of atherosclerosis in victims of sudden accidents. Their graphical results showed no correlation between serum cholesterol levels and severity of the disease at autopsy. Similarly, a more recent study (28) showed that when the expected positive correlation with age was removed there was no significant relationship between lesion severity and cholesterol levels. Thus, there appears to be a positive correlation between serum cholesterol levels and the incidence and severity of atherosclerosis; although, as shown, there is not complete agreement on this matter.

B. Cholesterol Biosynthesis

The biosynthesis of cholesterol is a very complex process of which most tissues are capable but which occurs most actively in the liver. The biochemical building unit for this synthesis is acetyl coenzyme A (acetyl CoA) which, through the series of initial reactions (Figure 1) is converted to the key intermediate mevalonic acid. This early portion of the entire biosynthetic sequence is most important to the present work. Following its production mevalonic acid is successively phosphorylated by ATP to afford 5-pyrophospho-3-phosphomevalonic acid (Figure 2). This compound decarboxylates and loses the 3-phosphate function to form 3-isopentenyl pyrophosphate which, in turn, is rapidly isomerized to dimethylallyl pyrophosphate.

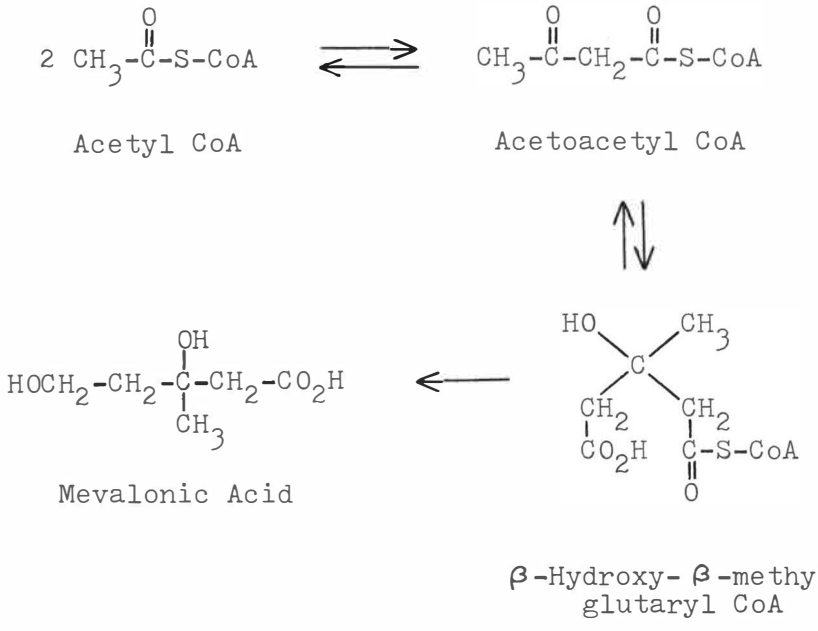


Figure 1: The biosynthetic conversion of Acetyl CoA to Mevalonic Acid.

Nucleophilic attack on dimethylallyl pyrophosphate by another molecule of 3-isopentenyl pyrophosphate leads to geranyl pyrophosphate. The repetition of this reaction with the new substrate leads to farnesyl pyrophosphate, two molecules of which couple to form squalene. The elegantly designed cyclization of squalene-2,3-epoxide (Figure 3) leads to lanosterol which, after several steps, is converted to cholesterol.

By following the conversion of 1-C^{14}-acetate to cholesterol in fractionated rat liver homogenates, Bucher and McGarrahan (29) found that neither mitochondrial, microsomal, nor supernatant fractions alone were capable of

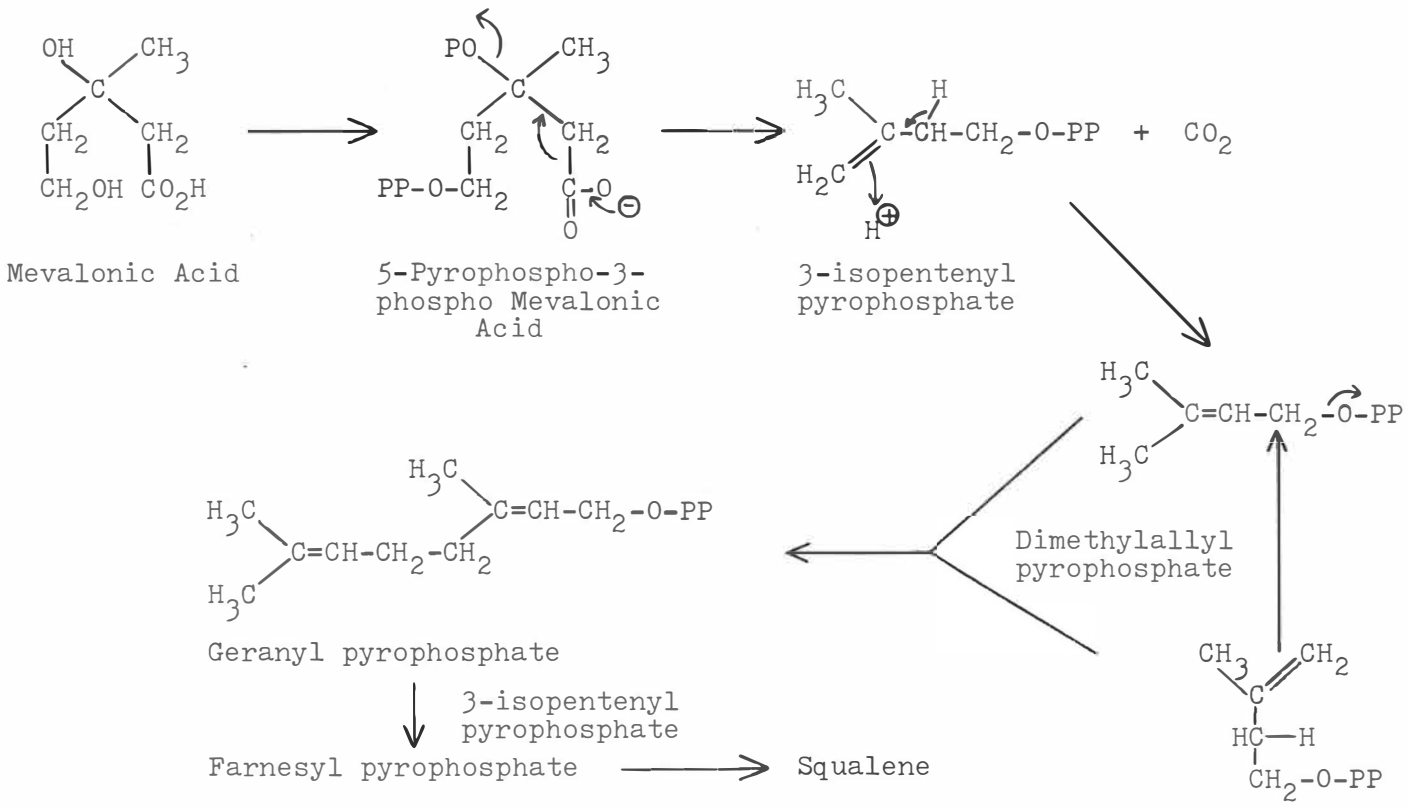


Figure 2: The biosynthesis of squalene from mevalonic acid.

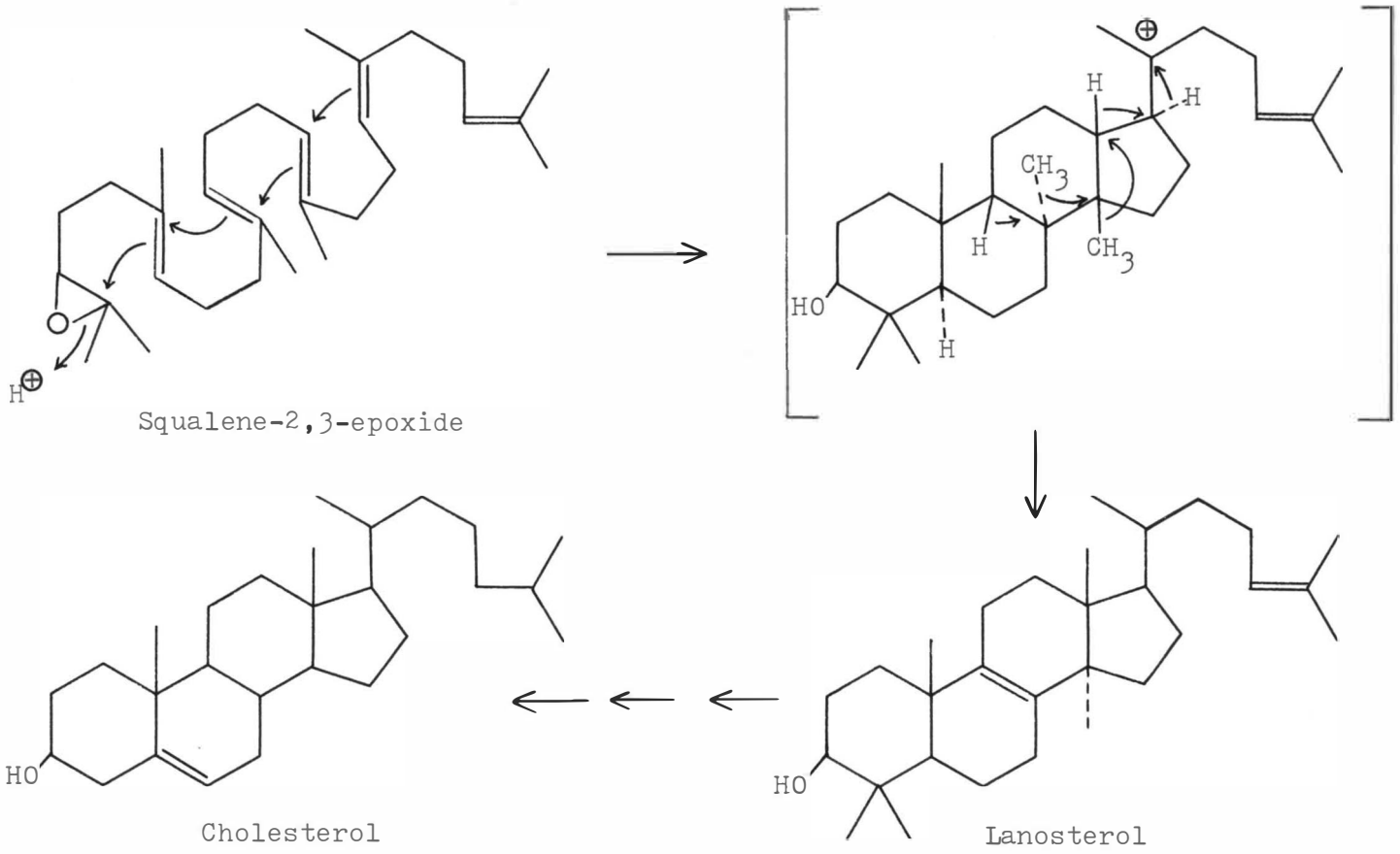


Figure 3: The cyclization of Squalene-2,3-epoxide.

cholesterol synthesis. Combination of the three fractions showed that most of the synthesis took place in the microsome, both in vitro and in vivo. Further localization of this process has shown that both the rough and smooth endoplasmic reticulum are capable of cholesterol synthesis from 2-C^{14}-mevalonic acid (30).

The earliest chemical studies of cholesterol biosynthesis were performed by Bloch and co-workers (31-34, 36). Initial attempts to determine the biological precursors of cholesterol consisted of feeding mice deuterioacetate, isolation of the biosynthesized cholesterol, and determination of its isotopic content by mass spectrometric techniques (31, 32). The first important result from these experiments was that deuterium labeled sodium acetate was incorporated into cholesterol. In addition, the quantity of label found made it possible to rule out fatty acids as the biosynthetic intermediates in sterol biosynthesis (31). These results stimulated further feeding experiments using various deuterium labeled acid salts. Of all the salts examined only acetate was capable of participation in cholesterol biosynthesis (32), and since the function of acetate as a metabolic intermediate was not then known this provided the first clue that coupling of very small molecules might be involved.

Conclusive proof that acetate is indeed the basic biosynthetic unit awaited double labeling experiments. Thus, incubation of rat liver slices with sodium acetate

labeled with deuterium in the methyl group and C^{13} in the carboxyl position showed that the isolated cholesterol contained both isotopes (33). Having established the basic unit, Bloch and his co-workers turned their attention to the quantitative aspects of label incorporation. Using variations of the original double label technique it was found that of the 27 carbon atoms of cholesterol, 15 were derived from the methyl group and 12 from the carboxyl carbon of acetate (34). Finally, using combinations of both chemical degradation and radioisotopic labeling experiments, the work of several investigators led to the elucidation of the origin of most of the carbon atoms in cholesterol (34-36) as illustrated in Figure 4.

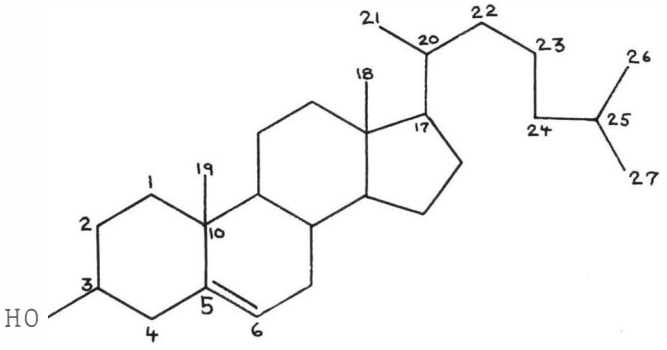


Figure 4: The chemical origin of carbon atoms of cholesterol (34-36). Positions numbered 1, 3, 5, 17, 18, 19, 21, 22, 24, 26, and 27 are derived from the methyl group of acetate; those numbered 2, 4, 6, 10, 20, 23, and 25 are derived from the acetate carboxyl carbon.

The above discussion of cholesterol biosynthesis is breif and emphasizes the early historical work and elucidation of the important function of acetate. The remaining aspects, especially conversion of lanosterol to cholesterol, are very complex and all of the mechanistic details are not clearly known. Since the present work is primarily concerned with the early portion of the biosynthetic pathway the remaining discussion will be centered on those reactions which mediate the conversion of acetate to mevalonic acid.

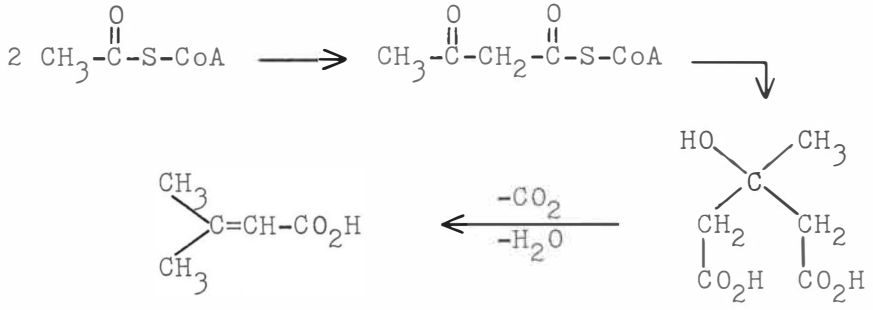
The early work of Bloch and other investigators indicates that the basic precursor or building block is acetate. Successful use of sodium acetate in incorporation studies using tissue slices or homogenates as well as in vivo experiments naturally led to the conclusion that acetate is probably employed without further biochemical modification. However, it is now known that intermediates occurring early in cholesterol biosynthesis (up to and including 3-hydroxy-3-methylglutaryl CoA) are all present as thioesters with coenzyme A (CoA). Light was shed on the question of whether coenzyme A is necessary in repeated use of acetate during cholesterol biosynthesis by the development of truly coenzyme A deficient rats (37). Correlation of reduced plasma and liver cholesterol levels with this deficiency proved the requirement for this cofactor in the conversion of acetate to cholesterol. This was the first indication that in acetate feeding experiments

the initial step of the synthetic pathway consists of the conversion of acetate to its CoA thioester. Under normal physiological conditions this step may not be required since the end product of other metabolic sequences (such as glycolysis and fatty acid degradation) is acetyl CoA.

Further investigation of the incorporation of labeled compounds as possible cholesterol precursors indicated the role of a four carbon compound. In vivo and in vitro experiments established that this compound was acetoacetate (39, 41); it therefore appeared that the second step of cholesterol biosynthesis was the condensation of two molecules of acetyl CoA to provide acetoacetyl CoA. Considerable confusion was caused by radioactivity incorporation data since some results were interpreted to show that prior to incorporation acetoacetate was equilibrated with a two carbon pool or was degraded in some other manner (38). This conclusion was based on the observation that incorporation of radioactive acetoacetate afforded cholesterol in which the labeled atoms were found widely distributed over the entire molecule. Similarly, the then unknown reversible nature of acetate condensation to form acetoacetate probably contributed to the confusion by causing label dilution. Other investigators (39) found direct incorporation of intact acetoacetate with no prior degradation to two carbon units.

The earliest indication of the nature of the third step of cholesterol biosynthesis was related to the

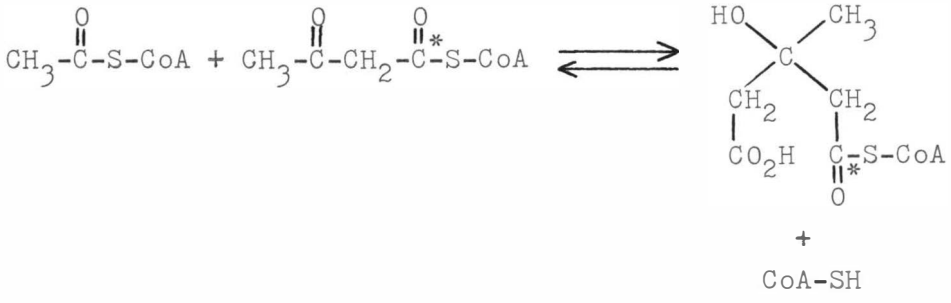
erroneous proposal that dimethylacrylic acid was a cholesterol precursor. This compound was probably obtained as an artifact by oxidation of dimethylallyl pyrophosphate during isolation. Rudney (40) proposed that this compound could be the result of dehydration and decarboxylation of β-hydroxy-β-methylglutaric acid (HMG) as shown in equation 1. To test this proposal HMG was biosynthesized from 2-C^{14}-acetate using a rat liver homogenate. Degradation and measurement of radioactivity from each carbon atom of the isolated compound showed very high C^{14} content at C2, C4, and C6. The most important aspect of this study was that HMG was correctly envisioned as being the product of the condensation of acetyl CoA and acetoacetate. Further investigation of this reaction showed that the four carbon precursor to HMG was definitely acetoacetyl CoA rather than free acetoacetate (41).



(eq. 1)

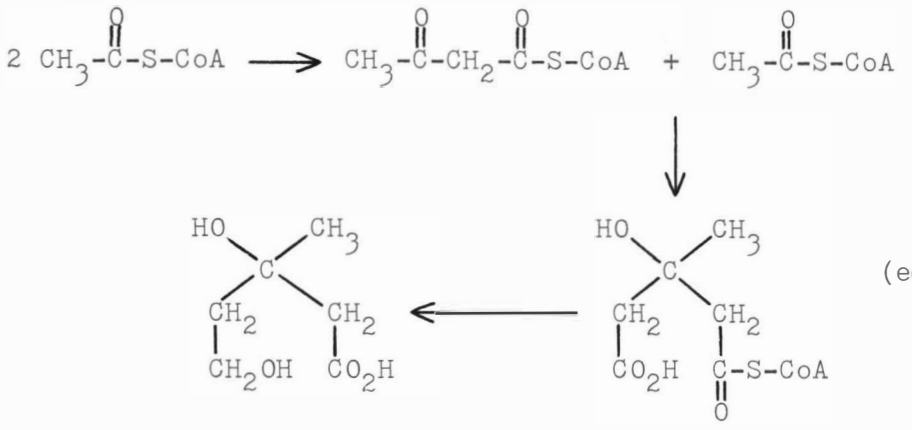
Additional study of the reaction (42, 43) revealed several important features of this step of cholesterol biosynthesis. The stoichiometry of the reaction showed that one mole of acetyl CoA reacts with one mole of

acetoacetyl CoA to provide a single mole of both β-hydroxy-β-methylglutaryl CoA (HMG CoA) and free coenzyme A and that the dicoenzyme A thioester was not formed. Appropriate labeling experiments (equation 2) conclusively proved that the free coenzyme A produced in the reaction was derived exclusively from acetyl CoA and not from acetoacetyl CoA. One of the most important aspects of this investigation was the proposal, based on structural similarities, that HMG CoA may be biochemically reduced to mevalonic acid which is an excellent substrate for cholesterol biosynthesis. It should be emphasized that up to and including biosynthesis of acetoacetyl CoA the pathways for fatty acid and isoprenoid syntheses are identical. Synthesis of HMG CoA is the branch point between these pathways. The biochemical alternatives for utilization of HMG CoA are either cleavage to acetoacetyl CoA and acetate and subsequent reentry into the two carbon pool or reduction to mevalonic acid and continuation into sterol biosynthesis.



(eq. 2)

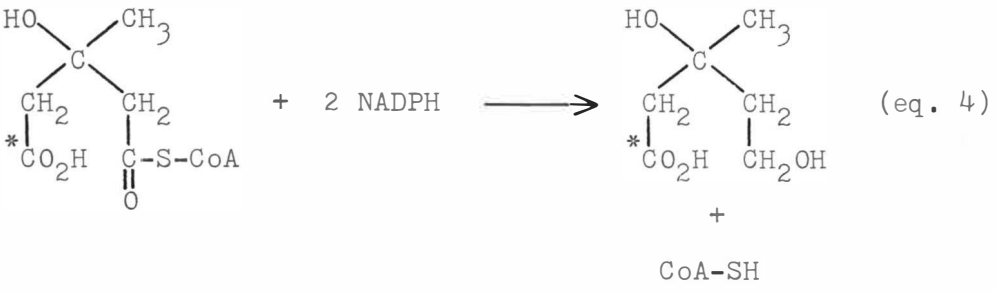
Important contributions to the elucidation of the final and most important early step of cholesterol biosynthesis were made by Ferguson et al. (44). Using yeast extracts these investigators established that HMG CoA was indeed reduced to mevalonic acid. These experiments completed the elucidation of the early sequence of reactions of cholesterol biosynthesis as shown in equation 3. This reduction



(eq. 3)

requires NADPH and the enzyme which mediates the reaction, HMG CoA reductase, accepts only the CoA thioester of HMG as substrate, not the free acid. Using 1-C^{14}-HMG CoA as substrate the resulting mevalonic acid was converted to its barium salt and pyrolyzed. The carbon dioxide generated from the resulting barium carbonate was found to be highly radioactive. This result proves that the free carboxyl group of HMG CoA remains intact during the reduction and therefore, the reduction takes place at the thioester carbonyl group (equation 4). Incubation of the enzyme with

2-C^{14}-mevalonic acid, NADP, oxidized glutathione, and free coenzyme A showed that the reaction is not reversible. The irreversibility of this reduction has important consequences on the entire series of reactions in early cholesterol biosynthesis. Thus, once the two carbon precursors have reached the mevalonic acid step they are not capable of reentering the two carbon pool and their progress toward incorporation into sterols becomes committed.



(eq. 4)

Since the reduction of HMG CoA to mevalonic acid requires two moles of NADPH it is tempting to suggest that the reaction takes place <u>via</u> an aldehyde intermediate. However, addition of unlabeled mevaldic acid or semicarbazide in an attempt to trap the aldehyde does not significantly lower incorporation of label into mevalonic acid (44, 62). Therefore, if an aldehyde is an intermediate in the reaction, it may occur as an enzyme bound species such as the thiohemiacetal of coenzyme A (44).

Concurrent with these studies Knauss and co-workers were investigating mammalian sterol synthesis. They independently discovered a rat liver preparation which was

capable of synthesizing mevalonic acid from acetate (45). These results established that the early steps of cholesterol biosynthesis in this mammalian system were identical with those shown in equation 3.

These early steps, from acetyl CoA to mevalonic acid, are very important with respect to the overall control of cholesterol biosynthesis. Many of the physiological phenomena known to alter sterol synthesis exert their effects at one of these steps. In this regard, HMG CoA reductase is the major site of action for several of these metabolic controls (46). For example, the detergent Triton WR 1339 and X-irradiation exert a stimulating effect on the conversion of acetyl CoA to both squalene and cholesterol but a much less noticable effect on the conversion of mevalonic acid to squalene or cholesterol (47). This suggests that the effect is exerted on one of the early reactions prior to mevalonic acid. The opposite is seen in response to fasting or cholesterol feeding. Conversion of acetate to cholesterol is greatly reduced, but conversion of mevalonic acid to cholesterol is altered to a much smaller degree. Again, the early steps are implicated as the sites of action. Studies of this type are the basis of suggestions that the early reactions of cholesterol biosynthesis include the rate limiting step for the entire pathway and that this step is probably located between acetoacetyl CoA and mevalonic acid (46, 47). Since these early hypotheses it has been found that the true rate

limiting step is the reduction of HMG CoA to mevalonic acid.

In the first example above, X-irradiation of rats causes an increase of cholesterol biosynthesis in the treated animals. The mechanism of this response is not clearly defined. It is known, however, that this process is not under adrenal control since adrenalectomized rats respond to X-irradiation just as do control animals (48). Similarly, hypophysectomy does not appear to alter the response although the results are not clear since the less drastic increase of cholesterol biosynthesis in these animals could have been due to poor condition of the animal, lack of pituitary hormones, or both (48).

The case of cholesterol feeding is more clearly illustrated. It is known that feeding cholesterol (0.5% of the diet) is associated with a marked decrease of cholesterolgenesis as measured by the incorporation of radioactive acetate (49). This is a specific inhibition of cholesterol synthesis and not a general metabolic liver depression since fatty acid and carbon dioxide production do not change. Decreased cholesterol biosynthesis in response to cholesterol feeding is complemented by an increase of biosynthesis after biliary obstruction or diversion (50, 51). Because cycloheximide administration blocks this rise in synthetic activity it is probable that changes in protein synthesis rates are responsible for these effects (50). By analogy this also indicates that cholesterol itself is

not a direct enzyme inhibitor.

As with cholesterol feeding, biliary diversion is a specific stimulus since the conversion of radioactive acetate to carbon dioxide or to fatty acids is not significantly changed. Restoration of the enterohepatic circulation of biliary diverted animals by intravenous or intrajejunal infusion of taurocholic acid, glycocholic acid, combinations of taurocholic and taurodeoxycholic acids, cholic acid, deoxycholic acid, or phospholipids did not block the increase of hepatic cholesterol biosynthesis observed after biliary diversion (51). Blockage of intestinal lymph flow, which caused interruption of the enterolymphatic circulation of endogenous cholesterol, also caused an increase of hepatic cholesterol biosynthesis which was quantitatively identical to that observed in response to biliary diversion. It was therefore concluded that the bile acids have no direct control on hepatic cholesterolgenesis; rather, they control absorption of cholesterol which is the true mediator (51). Localization of this effect shows that enhancement of HMG CoA reductase is the mechanism by which the observed increase of sterol synthesis takes place. Thus, cholesterol appears to participate in control of cholesterol biosynthesis by acting at the level of HMG CoA reductase; although, as discussed below, it does not interact directly with the enzyme. Further support for this regulatory mechanism was provided by the work of Bricker et al. (53). Using a desmosterol

suppression technique it was shown that cholesterol feeding caused an effective in vivo regulation of cholesterol biosynthesis. This control has also been found to operate in man (52) where the activity of the control system varied with the levels of dietary cholesterol.

In feeding experiments of relatively short duration the site of control which dietary cholesterol exerts upon cholesterol biosynthesis is located at the reduction mediated by HMG CoA reductase. However, there are some indications that under varied feeding conditions other less important control sites may exist after the formation of mevalonic acid. These additional sites are difficult to detect under normal experimental conditions but if cholesterol feeding is extended for long periods of time they become significant. By appropriate labeling experiments it has been shown that conversion of squalene is unaltered but conversion of farnesyl pyrophosphate to cholesterol is reduced to 3 to 35% of control values in response to long term cholesterol feeding of rats (54). This site alone does not account for the magnitude of depression of conversion of mevalonic acid to cholesterol observed in these same experiments. This suggests that an additional control site exists between mevalonic acid and farnesyl pyrophosphate. The contribution of these postmevalonate sites increases with feeding times until complete suppression of cholesterol biosynthesis is achieved. Thus, HMG CoA reductase is the most important regulatory site under normal

and most experimental conditions but when blockade approaches completeness as with cholesterol feeding for extended periods, the postmevalonic acid sites become important.

Although most studies of cholesterol biosynthesis are concerned with hepatic synthesis, extrahepatic tissues are also capable of cholesterol synthesis. In a comprehensive study of synthesis and control characteristics of various rat tissues, Dietschy and Siperstein (55) found that, per gram of tissue, the liver, ileum, transverse colon, and stomach exhibited the highest rates of incorporation of acetate into cholesterol. The esophagus, jejunum, testis, lung, adrenal, skin, kidney, duodenum, and spleen showed 2-10% of the hepatic rate while smooth muscle, skeletal muscle, and brain had incorporation rates less that 0.6% that of hepatic rates. Even though several organs are capable of cholesterol biosynthesis the ability to control the process by cholesterol feeding remains unique to the liver. In these experiments (55) cholesterol feeding was of six weeks duration. Additionally, fasting for 48 hours depressed cholesterol biosynthesis only in the liver.

Of all the extrahepatic tissues, cholesterol biosynthesis has been most thoroughly examined in the gastrointestinal tract. The rate of synthesis varies along the length of the intestine, with ileum being highest (56, 57). This variation extends to the type of tissue examined; intestinal smooth muscle and villi are poor in cholesterol

biosynthetic ability and the highest rates are observed in crypt cells. As in liver, biliary diversion increases intestinal sterol synthesis but does not alter other synthetic pathways. This effect has been attributed to increased activity of HMG CoA reductase (56). Restoration of the enterohepatic circulation by infusion of whole bile or solutions of bile acids suppresses synthesis; however, infusion of bile from which bile acids have been removed shows no effect. This data can be interpreted to suggest that either bile acids participate in control of intestinal cholesterol biosynthesis or that bile acids play a secondary role by facilitating entry of cholesterol into the intestinal mucosa where it is the true mediator of regulation. The second possibility may be doubtful because cholesterol feeding appears to have no effect on intestinal synthesis (56). Similarly, intestinal synthesis is relatively resistant to the effects of fasting seen in hepatic tissue. It can be concluded that although liver and intestinal sterol synthesis are physiologically controlled, the mechanisms of control in the two tissues are considerably different.

In man, the results are similar to those found in animals (58). The major difference is that fasting for 48 hours reduces human intestinal cholesterol biosynthesis to 50% of control values (58). As with animals, cholesterol feeding does not inhibit cholesterol biosynthesis in human intestine. The gastrointestinal tract is therefore

probably an important source of endogenous cholesterol under conditions of high cholesterol intake when liver synthesis is drastically decreased. Finally, when the enterohepatic circulation is interrupted human intestinal tissue responds with an increased level of cholesterol biosynthesis. This suggests that man has a bile acid associated control system similar to the one observed in animals (58).

Hepatic cholesterol biosynthesis exhibits a diurnal rhythm with its maximum occurring around midnight at levels four to ten times higher than the minimum, which occurs at noon (59-61). Although feeding bile acids inhibits biosynthesis in rats there is no correlation between the diurnal rhythm and hepatic bile acid concentrations. Therefore, the bile acids are not the cause of the rhythm (59). Rather, since HMG CoA reductase has been previously implicated as a major control point for the entire sequence of reactions and since factors capable of altering the rhythm appear to act through this enzyme it is believed that the cyclic changes are due to similar changes at the enzyme level. Administration of cycloheximide abolishes the diurnal rise during the dark period (59). This is conclusive evidence that the nature of this rhythm is based on enzyme synthesis rather than on changes of enzyme activity.

Several experimental manipulations have effects on this cyclic rhythm and, <u>in vitro</u>, these can be studied by measuring the incorporation of radioactive acetyl CoA or

mevalonic acid into cholesterol. Fasting, which normally reduces the magnitude of hepatic cholesterol synthesis, also effects the diurnal rhythm of synthesis (60). Under fasting conditions the cyclic nature of the rhythm is maintained but the magnitude of synthesis is greatly reduced. Also, after bilateral adrenalectomy the rhythm is abolished, but the level of cholesterol synthesis is maintained at approximately the maximum level observed during dark periods. Adrenalectomy does not alter the biosynthesis of cholesterol as measured from mevalonic acid incorporation. This once again implicates the important enzyme HMG CoA reductase as being the site through which synthetic alterations are expressed (60). The regulator of this rhythm cannot be norepinephrine since this agent causes enhancement of cholesterol synthesis. A possible regulator is corticosterone (60) since this adrenal steroid also is subject to diurnal variations. The diurnal variations of corticosterone levels can be abolished by cutting the afferent nerves to the medial basal hypothalamus or by maintaining the experimental animal in continuous light. If corticosterone is the mediator it would be expected that these conditions would destroy diurnal variations of cholesterol synthesis also. A similar experiment will be discussed in a later section.

The diurnal rhythm has also been demonstrated under <u>in vivo</u> conditions in rat intestinal tissue as well as in

liver (61). Reversal of light-dark cycles is accompanied by a similar phase shift of the biosynthetic rhythm. Since rats consume most of their daily food ration during dark periods and the 2.5 hours preceding onset of darkness it is suggested that the true stimulus of the rhythm is food consumption rather than darkness. To test this suggestion rats were allowed access to food under normal lighting conditions (lights on 6 AM to 6 PM) only from 9 AM to 1 PM. This caused a shift of daily rhythm and the new maximum of cholesterol synthesis activity occurred at 6 PM (61).

C. β-Hydroxy-β-methylglutaryl Coenzyme A Reductase

Early definitive studies on the reduction of HMG CoA were done by Durr and Rudney (62). Using an enzyme preparation derived from bakers' yeast these workers described properties of the reaction which were later found to be in common with the same enzyme derived from mammalian sources. As the enzyme was purified beyond a specific activity of 1.0 it became increasingly unstable and required thiol protecting reagents to maintain the activity. This, as well as the subsequent finding that reduction of the substrate was completely inhibited by p-hydroxymercuribenzoate, demonstrated the enzymatic requirement for free thiol groups. For each mole of HMG CoA reduced two moles of NADPH are consumed and one mole each of free coenzyme A and mevalonic acid are produced (equation 4). The enzyme was

found to be specific for HMG CoA only and acyl transfer or phosphate transfer were not required. Additionally, the reaction was demonstrated to take place irreversibly. The irreversible nature of the reaction is reasonable since thioester bonds are high energy sources and their reductive cleavage is accompanied by free energy loss of approximately 8200 calories per mole (62). The monodirectional biosynthetic flow at this reaction is one of the early stimuli which suggested the regulatory nature of this enzyme. Finally, the continuing question of the identity of possible reaction intermediates was opened by this investigation (62). Inability to inhibit reduction of HMG CoA by the addition of semicarbazide or the semicarbazone of mevaldic acid was evidence that the free aldehyde was not involved as an intermediate.

Reduction of HMG CoA to mevalonic acid is the first unique step as well as the rate limiting step of polyisoprenoid biosynthesis (63, 64). Early suggestions that this unique position is related to possible regulatory functions of the enzyme were made by Siperstein (64). Using incorporation experiments and a rat liver preparation it was shown that cholesterol feeding exerted its effects on this enzyme. Conversion of squalene to cholesterol or of mevalonic acid to squalene were not depressed by cholesterol feeding and therefore it was reasoned that the site of synthesis depression must be prior to mevalonic acid. Acetoacetyl CoA is a precursor for the synthesis of long

chain fatty acids as well as for cholesterol. Similarly, HMG CoA is a precursor not only of cholesterol but also of ketone bodies, acetoacetate and β-hydroxybutyric acid. Therefore, if cholesterol feeding exerts its effects at the conversion of acetate to acetyl CoA, acetyl CoA to aceto-acetyl CoA, or of acetoacetyl CoA to HMG CoA a block of fatty acid and ketone body synthesis would be observed along with a block of cholesterol biosynthesis (Figure 5). Since cholesterol feeding is known to alter only choles-terol biosynthesis it was concluded that the site of this action must be HMG CoA reductase (64).

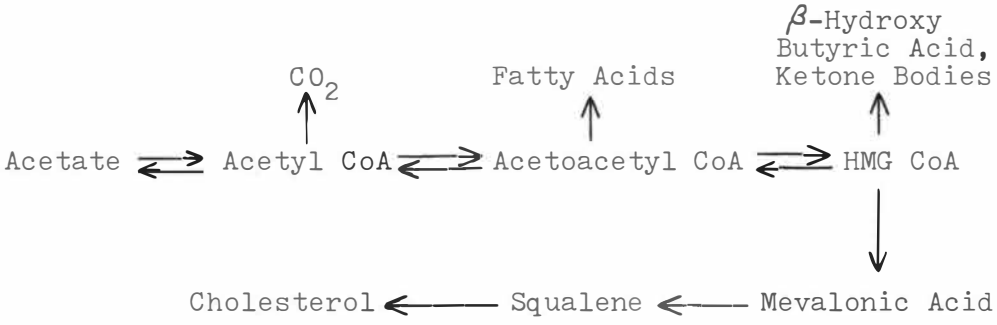


Figure 5: Alternate pathways for the early intermediates in cholesterol biosynthesis, after Siperstein (64).

The elegant efficiency of placing a regulatory site at this position is striking; synthesis of mevalonic acid is the first reaction subsequent to the final branching point for alternate pathways of these early intermediates. A regulatory site located closer to cholesterol would cause

unnecessary synthesis of intermediates and would create the problem of disposal of those intermediates. With the regulatory site located at mevalonic acid synthesis the latter problem does not exist since the earlier reactions are reversible and alternate synthetic pathways are available to the intermediates. On the other hand, if the regulatory site is located prior to mevalonic acid synthesis, the production of other important metabolic products would be altered along with the normal regulation of cholesterol biosynthesis.

Mammalian hepatic HMG CoA reductase is bound to the endoplasmic reticulum and therefore is found in microsomes on isolation. Centrifugal fractionation of rat liver microsomes has shown that the smooth and "smooth" smooth endoplasmic reticulum together contain the majority (81%) of enzyme activity while the rough membrane fraction accounts for only 14% of the activity (65). The observation of a small amount of enzyme activity in the polysome fraction is reasonable since this is the most probable site of enzyme synthesis. The subcellular localization of this reductase is important with respect to the location of HMG CoA condensing and cleavage enzymes. The latter two enzymes, which catalyze the forward and reverse reactions shown in equation 2, respectively, are located in rat liver mitochondria (66). Since the cleavage enzyme is approximately ten times more active than the condensing enzyme it might appear that net cholesterol biosynthesis would be

considerably hampered, if not impossible. Compartmentalization of these enzymes explains why liver tissue is capable of overcoming this apparent anomaly. Being confined to mitochondria, the cleavage enzyme destroys only mitochondrial HMG CoA. Small amounts of condensing enzyme attached to microsomal membranes allows all of its product, as well as any HMG CoA which might leave the mitochondria, to be available to the reductase (66). Thus, it can be seen that because of this compartmentalization the earliest steps of cholesterol biosynthesis, where intermediates are employed in alternate pathways, take place in the mitochondria. The unique step, mevalonic acid synthesis, and reactions subsequent to it take place on the endoplasmic reticulum.

Even though HMG CoA reductase is bound to the endoplasmic reticulum membranes the enzyme has been solubilized and refined to various degrees of purity (67-70) allowing study of several of its properties. The earliest successful attempt at solubilization provided an acetone powder derived from rat liver (67) and was not an entirely satisfactory preparation. Using deoxycholate to remove the enzyme from membranes Kawachi and Rudney (68) obtained a preparation from rat liver which allowed the determination of some physical and kinetic properties. The molecular weight of the purified enzyme was measured in the range 217,000 to 226,000 and the previous report of a thiol requirement (62) for the enzyme was substantiated and

confirmed with this purified preparation.

Recent success in solubilizing the reductase from rat liver microsomes illustrates the delicacy with which it must be handled. Taurocholic acid, pH change, presence or absence of salts, and quick freezing methods all proved unsuccessful in removing the enzyme from endoplasmic reticulum membranes (69). However, a slow freeze-thaw cycle (8-10°C per minute to -50°C) provided a soluble enzyme preparation containing 80% of the activity of the original rat liver microsomal pellet. Further purification of this preparation is possible by ammonium sulfate precipitation followed by agarose gel chromatography (69). Similarly, the most recent attempts also emphasize the delicate nature of the enzyme. Thus, incubation of rat liver microsomal pellets with potassium chloride solutions or suspension of a previously frozen pellet in glycerol provided active reductase preparations (70). These gentle conditions removed only approximately 25% of the microsomal protein. Also, the electron microscopic appearance of microsomes after removal of the reductase showed that they remained intact throughout the procedure. This ease of removing the enzyme without either disrupting the membrane or apparently breaking covalent bonds indicated that the reductase was peripherally located on the surface of, rather than being an integral component of the microsomal membranes. This is in agreement with the observation that HMG CoA reductase is synthesized and destroyed <u>in vivo</u> much

faster than the remaining microsomal protein (93).

As with most other chemical reactions which take place in living systems the reduction mediated by HMG CoA reductase is stereospecific. Using HMG CoA and the hemithioacetal of mevaldic acid as substrate for the yeast enzyme, Dugan and Porter (71) demonstrated that only A-side, or (4R)-4-H^3-NADPH, incorporated the tritium atom into mevalonic acid. If the two proton transfers involved in this reduction took place with opposite stereochemistry, that is, one transfer from the 4R side and one from the 4S side of NADPH, the maximum theoretical yield of 5-H^3-mevalonic acid would be 50%. However, with HMG CoA as substrate 78% transfer of tritium was observed. Similarly, using the hemithioacetal of mevaldic acid as substrate showed a high transfer of the isotope. These data are conclusive evidence that both proton transfers from NADPH take place stereospecifically and from the 4R side of the pyridine ring. Other investigators (72, 73) have confirmed this finding. Blattmann and Rétey (72) found that the yeast enzyme acted stereospecifically with respect to C3 and C5 of the substrate. In each case reduction of the monothiohemiacetals of (3RS)-mevaldate with coenzyme A and (3RS)-mevaldate with (R)-pantetheine as well as (3RS)-mevaldate alone, using (4R)-4-H^3-NADPH provided only (3R, 5S)-5-H^3-mevalonic acid (Figure 6).

As discussed previously, free mevaldic acid is probably not an intermediate in this reduction (44, 62). Beyond

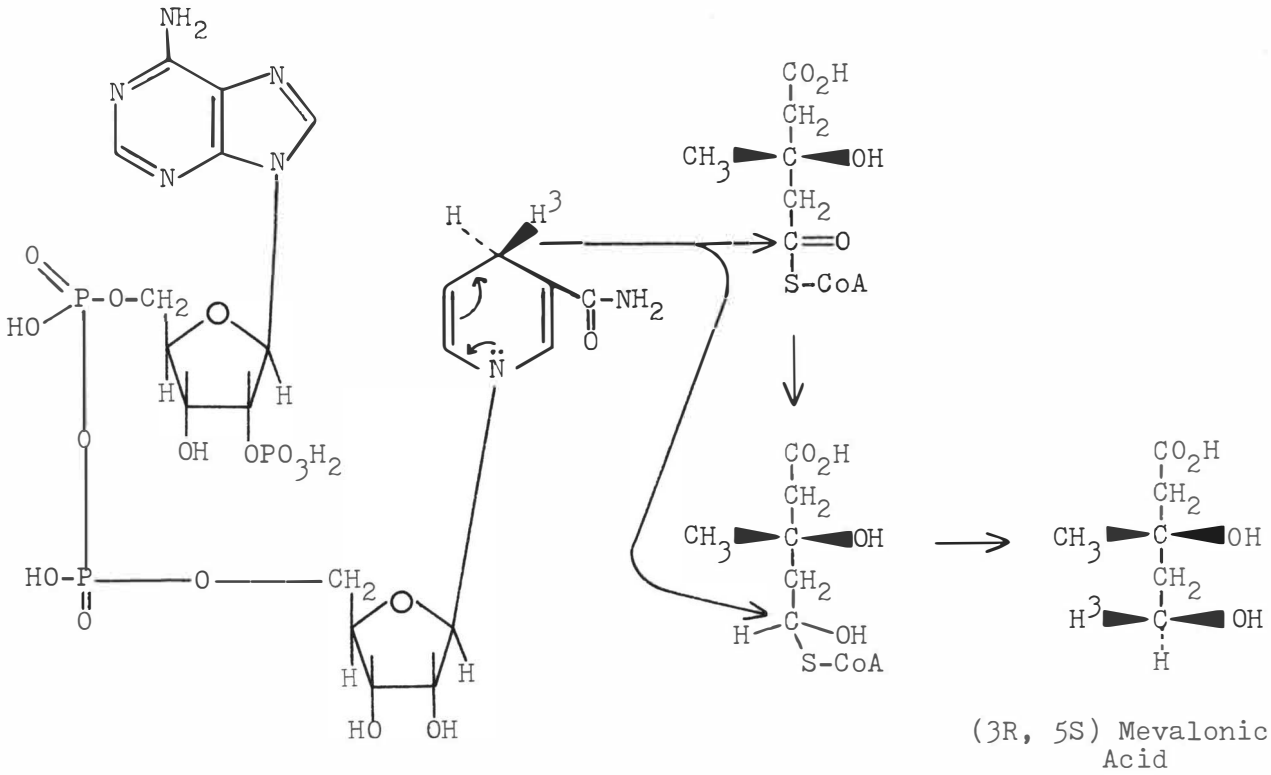


Figure 6: The stereospecific reduction of β-Hydroxy-β-methylglutaryl CoA.

this fact, the question of an intermediate for this two step reaction is clouded by conflicting experimental results. Brodie and Porter (74) were able to trap radioactive mevaldic acid after incubating a rat liver microsomal preparation with 1-C^{14}-acetate, however, the amount of mevaldic acid detected was not large enough to justify the suggestion of a free aldehyde intermediate. Therefore, it was proposed that HMG CoA is first reduced to an enzyme bound thiohemiacetal which is hydrolyzed to give enzyme bound mevaldic acid. This would explain the small amount of mevaldic acid detected as being a result of leakage from the surface of the enzyme.

These data are in accord with the later experiments of Kirtley and Rudney (75). From kinetic studies with a yeast enzyme preparation these investigators found that mevalonic acid is an uncompetitive inhibitor with respect to NADPH. Uncompetitive inhibition by the product was interpreted to mean that the product was bound to a different form of the enzyme from that which bound the substrate. A possible mechanism was proposed which involves an enzyme bound mevaldic acid (Figure 7).

Rétey and co-workers investigated the possibility of enzyme bound intermediates in the yeast system (76). Using radioactive coenzyme A it was found that the reductase did not participate in acyl exchange as illustrated in equation 5. This indicates that there is probably no transfer of HMG to a free SH group on the enzyme surface

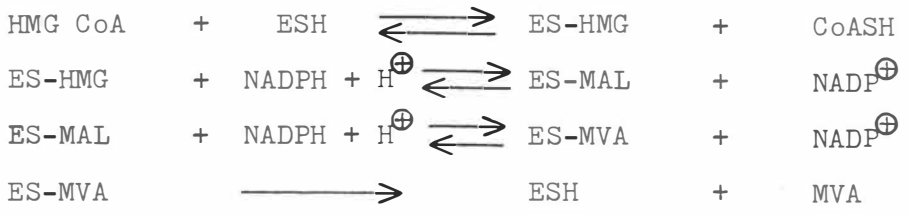


Figure 7: Mechanism proposed by Kirtley and Rudney (75) illustrating enzyme bound mevaldic acid. E = enzyme, MAL = mevaldic acid, MVA = mevalonic acid.

and, therefore, no formation of enzyme bound intermediates as proposed by Kirtley and Rudney (75) and by Brodie and Porter (74). This enzyme preparation did not reduce HMG-S-pantetheine, but (RS)-mevaldic acid-pantetheine hemithioacetal was readily reduced almost as fast as normal substrate. This observation indicates that conversion of substrate to a hemithioacetal and reduction of this hemithioacetal to mevalonic acid occur at two different sites on the enzyme surface. The first proposed site would be strictly specific for HMG CoA as substrate while the latter site would accept mevaldate CoA hemithioacetal. It is clear that further experiments are necessary to fully elucidate the true nature of possible intermediates in this reaction, and specifically, whether these two sites actually exist and, if so, whether they represent physically distinct regions of the enzyme, conformational changes taking place at the active site, or both.

The developmental pattern of HMG CoA reductase in rats has been studied by Rodwell et al. (77, 78). In general, enzyme activity rises very rapidly just prior to birth then sharply falls after birth to a level near that of adults. This level is maintained for eight days then declines precipitously. On the third day after weaning enzyme activity overshoots adult levels threefold and slowly declines until adult levels are reached in the second week after weaning. It is interesting to note that throughout development cholesterol synthesis parallels these variations in enzyme activity, indicating that the reductase is the rate determining step of cholesterol biosynthesis during development.

The drastic fall of activity prior to weaning is not due to cholesterol in suckling milk. This was demonstrated by the observation that hepatic cholesterol levels change very little during suckling; both free and total hepatic cholesterol levels remain within $\pm$ 10% of adult levels throughout development (77). Premature weaning caused a rise in activity as did normal weaning and late weaning prevented this rise. Thus, it appears that the rise of activity associated with weaning is due to dietary changes which take place at weaning.

Evidence for the presence of an inhibitor of the reductase in suckling rat liver was observed (77). Incubation of adult rat liver microsomal enzyme preparations with the 7,000 x g and 50,000 x g supernatant from suckling liver homogenates caused a 20% reduction of adult activity.

This depression was not observed with adult supernatant solutions. The inhibitor could be precipitated by ammonium sulfate and was nondialyzable but has not been characterized. The initial rise of enzyme activity from the low suckling values to adult levels was not blocked by cycloheximide administration and therefore must be due to relief of an inhibition of the enzyme which takes place at weaning, rather than by alterations in protein synthesis. Thus, the developmental pattern of this enzyme seems to be controlled by dietary factors which appear to regulate enzyme synthesis as well as by an uncharacterized hepatic inhibitor of the reductase.

Most of the previously discussed factors which alter or regulate cholesterol biosynthesis have been shown to act by exerting their influence on HMG CoA reductase. As a body, this work provides proof of the important rate limiting and regulatory functions of this enzyme. Many of these studies have been approached by measuring changes in the degree of incorporation of one intermediate into another or into cholesterol itself in response to some physiological or experimental condition. A note of caution has been raised concerning this method (79) since it assumes that changes of incorporation rates directly reflect changes of rates of biosynthesis.

Rates of incorporation of radioactivity into a specific product are not necessarily equal to rates of synthesis of that product because of possible dilution of

the radioactive precursor by endogenously synthesized precursor. For example, using rat liver microsomes White and Rudney (79) found that the specific activity of labeled acetoacetate was only 16% of what it should have been if C^{14}-acetyl CoA were the only precursor. This effect was due to formation of acetoacetate by endogenous synthesis and dilution of the labeled pool. These results cast doubt on experiments showing HMG CoA reductase to be the control point for cholesterol biosynthesis since this work is based on incorporation of labeled substrate into a product. Changes of incorporation into a product are an accurate measure of an alteration of the synthesis of that product only if the specific activity of the precursors remains unchanged. Thus, in the present case of cholesterol synthesis when some experimental condition alters incorporation of acetyl CoA but not of mevalonic acid into cholesterol the alternate explanation to considering HMG CoA reductase as a regulatory reaction is that unlabeled substrate from endogenous sources may have entered the pathway prior to mevalonic acid. This would reduce the specific activity of precursors and therefore make it appear that synthesis has been inhibited. If the experimental condition enhanced fatty acid oxidation or glycolysis, the pool size and therefore the specific activity of acetyl CoA would change.

Possible solutions to this problem include removal of endogenous substrate pools from the test system or using

such large quantities of radioactive substrate that endogenous contributions would be insignificant. The former solution is not feasible while the latter would be satisfactory only for single step reactions and the high concentrations of substrate necessary may inhibit other reactions. White and Rudney have solved this problem in a general manner by developing a method for the direct measurement of acetoacetate; since this intermediate is related to both HMG CoA and mevalonic acid, the necessary specific activities could be calculated (79). Similarly, these investigators developed three systems for the direct assay of HMG CoA reductase as well as a method which cleanly separates and measures HMG and mevalonic acid. The results showed that for the early steps of cholesterol biosynthesis the tacit assumption of equality of label incorporation and synthesis rates was accurate; therefore previous experiments indicating the regulatory nature of the reductase remain valid. Thus, after unveiling this potentially serious problem White and Rudney (79) have provided a satisfactory general solution.

Among all the experimental conditions used to study this enzyme, cholesterol feeding is perhaps the best known. As described previously, dietary cholesterol reduces cholesterol biosynthesis and it has been found that this regulatory action takes place at the site of HMG CoA reductase (63, 78, 80-83). The reductase is always the most important site of inhibition, but if cholesterol feeding is of

extended duration other sites also contribute to the overall blockade. The inhibition obtained by feeding cholesterol is drastic; short periods provide 80-90% or more depression of cholesterol biosynthesis while extended feeding periods are associated with complete suppression of synthesis.

Most evidence indicating that HMG CoA reductase is the true regulatory site for cholesterol biosynthesis is of an indirect nature. Siperstein and Fagan (81) developed gas chromatographic analytical techniques which allowed separation and measurement of HMG and mevalonic acid. As in other systems cholesterol feeding caused depression of synthesis by more than 95% as measured from rat liver microsomes while there was no effect on the condensing enzyme. Direct measurement showed that HMG levels were normal, but mevalonic acid levels were severely depressed in cholesterol fed animals. The depression of mevalonic acid levels was sufficient to account for the corresponding depression of cholesterol synthesis. These data substantiate previous indirect evidence that the reductase is the site of action during cholesterol feeding and the analytical technique used makes this work the first direct observation of this fact.

Using the computational technique (79) which avoids the assumption that incorporation rates are equal to synthesis rates White and Rudney also showed that in rat liver microsome preparations cholesterol feeding caused depression

of reductase activity by more than 90% (83). When a soluble system was used small degrees of depression of HMG CoA condensing enzyme were observed under cholesterol feeding conditions. This work agrees with the fact that the major effect is on HMG CoA reductase but also indicates that under conditions where substrate availability for the reductase is limiting the slight inhibition of condensing enzyme may become important.

The mechanism of the clearly demonstrated lowering of HMG CoA reductase activity caused by dietary cholesterol has been the object of much investigation. Possible mechanisms include (78, 82): 1) direct inhibition of the enzyme, 2) allosteric inhibition of the enzyme, 3) changes of the rate of enzyme synthesis by interfering with mRNA synthesis, destabilization of polysomes, or interference with incorporation of the completed enzyme into the endoplasmic reticulum, or 4) increasing the rate of dissociation of the enzyme from the endoplasmic reticulum or enhancing the degradation rate of the enzyme, or both. The first possibility may be excluded since cholesterol or some other soluble compound does not act as a direct inhibitor (82). This conclusion was derived from the observations that albumin or propylene glycol suspensions of cholesterol, as well as cholesterol rich low and very low density lipoproteins from livers of both normal and cholesterol fed animals did not alter reductase activity in vitro (78). Similarly, mixing experiments where liver

preparations from cholesterol fed animals were added to normal preparations showed no alterations of activity and therefore the absence of a soluble inhibitor. The second possible mechanism may also be excluded. Although the total activities of the enzyme derived from normal and cholesterol fed animals are vastly different their kinetic properties are so similar that the reductase cannot be an allosteric enzyme (78). It is therefore evident that dietary cholesterol is not a true feedback inhibitor of cholesterol biosynthesis.

The apparent inhibitory effect of cholesterol on HMG CoA reductase is actually one of altering the amount of enzyme rather than the activity of the reductase (78, 82). Experiments involving study of the diurnal rhythm of this enzyme indicate that dietary cholesterol acts to alter the rate of rise of enzyme synthesis while the normal rate of degradation is maintained. Thus, the true mechanism of dietary cholesterol on cholesterol biosynthesis is an unknown effect which alters synthesis rates of HMG CoA reductase.

As with cholesterol feeding, severe lowering of HMG CoA reductase levels is caused by fasting (63, 78, 80, 83), a physiological condition which is apparently opposite to that of cholesterol feeding. This effect is expressed at the reductase step and appears to be associated with changes of enzyme synthesis. The mechanism of this phenomenon is not understood.

The major site at which the effects of cholesterol feeding and fasting are expressed is HMG CoA reductase. In an attempt to determine if other secondary regulatory sites exist Slakey et al. (84) studied the effects of fasting and refeeding on the seven enzymes which mediate the conversion of HMG CoA to squalene. Sterol synthesis from C^{14}-acetate and C^{14}-mevalonic acid was depressed by fasting and rose in response to refeeding. For each enzyme examined except mevalonic kinase and phosphomevalonic kinase, the total activity fell during fasting; however, with respect to HMG CoA reductase the magnitude of these depressions was small. It was concluded that during most of the fasting and refeeding periods the overall rate of cholesterol biosynthesis was controlled by the amount of HMG CoA reductase. The time course for conversion of acetate to digitonin precipitable sterols was parallel to that of reductase activity, and total activity of the reductase was considered sufficient to account for the observed rate of sterol synthesis. This conclusion was based on studies of only the initial and final components of the entire sterol biosynthetic pathway. Kinetic analysis indicated that the enzymes in this seven membered segment of the pathway are not coordinately controlled and that the flux of material through this portion of the pathway was not well explained by considering HMG CoA reductase as the sole regulatory step. Thus, it was concluded that there may be two additional regulatory sites between mevalonic acid and squalene.

This conclusion was in agreement with the work of Gould and Swyryd (54) who postulated the existence of two secondary regulatory sites between mevalonic acid and squalene. The additional sites detected by Slakey et al. (84) may be identical to those proposed by Gould and Swyryd and, like the latter, may become important only under certain conditions.

As previously discussed for cholesterol biosynthesis, with very little emphasis placed on the role of the rate limiting reaction, bile acids alter the conversion of acetate to cholesterol, their presence being associated with decreases and absence with increases. It has been shown that these effects are due to changes in the level of HMG CoA reductase (78, 85, 86) although the exact mediator of these changes is a subject of considerable disagreement. Using rat liver microsomes Hamprecht et al. (85) showed that bile acids inhibited the reductase in vitro and taurochenodeoxycholate and taurodeoxycholate were the most efficient inhibitors. Since the total bile acid concentration in rat liver is only approximately 0.3 mM, in vitro inhibition of cholesterol biosynthesis by bile acids is probably not due to actual inhibition of the enzyme because the above experiments were done with concentrations of bile acids greater than the physiological concentration. Similarly, this inhibition was neither fully reversible nor fully irreversible, indicating that bile acids were not allosteric inhibitors of the reductase. Therefore, it was

proposed that *in vitro* effects of bile acids on this enzyme were due to a nonspecific detergent action on the membrane bound enzyme (85). *In vivo* experiments (85) indicated that bile acids altered synthesis of the enzyme and that their *in vitro* activity did not accurately reflect *in vivo* activity.

Two possibilities for the mechanism of bile acid inhibition of cholesterol biosynthesis were considered: The bile acids either altered synthesis, degradation, or both, of HMG CoA reductase or they enhanced absorption of cholesterol which was the true mediator (86). When absorbed in the gut cholesterol must pass through the thoracic duct before reaching the liver. On the other hand, bile acids reach the liver directly after absorption *via* the portal vein. For these reasons, in rats with cannulated thoracic ducts cholesterol is eliminated after absorption and never reaches the liver. This allows the study of the effects of bile acids independent of any effects related to cholesterol absorption. Cholic acid feeding of rats with this lymphatic diversion depressed hepatic HMG CoA reductase. Thus, Hamprecht *et al.* (86) believe that bile acids exert their effect on cholesterol biosynthesis by interfering with the synthesis of HMG CoA reductase. These results are in direct disagreement with the work of Weiss and Dietschy (51) who, by experiments with bile fistula rats, claim that bile acids alter cholesterol biosynthesis only by improving intestinal absorption of cholesterol.

As with many biochemical processes, hormones influence HMG CoA reductase but their effects have been studied to a much less comprehensive extent than dietary and drug effects. Thyroid hormones were found to alter reductase levels. Using isolated perfused rat liver, Gries et al. (87) studied the effects of these hormones on levels of the reductase as well as of other enzymes and discovered that the level of reductase present was a function of the concentration of thyroid hormone present. It was observed that after perfusion with DL-thyroxine, enzyme levels increased and paralleled cholesterol biosynthesis. After thyroidectomy, HMG CoA reductase levels fell until activity was barely detectable; however, this activity could be reestablished by triiodothyronine. Similarly, with rat liver microsome preparations, injection of I^{131} to suppress thyroid function of the test animal caused the reductase activity to be lowered to 42% of control values (88). Thyroidectomy caused a fall in reductase levels to 35% of normal levels, in agreement with the experiments of Gries et al. (87). Finally, hypothyroid animals were injected with 3,3',5'-triiodothyronine, killed, and HMG CoA reductase activity was assayed in the livers. Until 30 hours after injection reductase activity remained in the range typical of thyroidectomized animals. Thereafter, activity rose rapidly until 45 hours after injection when normal reductase activity was achieved and maintained for the remainder of the experiment. The 30 hour latent period in

these experiments indicated that triiodothyronine was stimulating enzyme synthesis rather than having a direct effect on the enzyme itself (88). It is possible that this activity of thyroid hormones is due to their general stimulation of metabolic functions, although later experiments show this to be unlikely.

Another hormone which alters HMG CoA reductase activity is insulin. It has been recently demonstrated that rat liver microsomal reductase activity increased two to threefold with respect to controls after subcutaneous insulin administration (89) and diabetic rats exhibited an even larger increase of activity. For both the normal and diabetic animals, the observed increases of activity corresponded to increases of acetate incorporation into cholesterol. Glucagon, the physiological insulin antagonist, completely blocked these enhancements when administered two hours prior to insulin. These experiments are another case in which the effect of an experimental condition on HMG CoA reductase is believed to have been caused by increase of activity due to the stimulation of synthesis of new enzyme. In similar experiments it has been found that the reductase activity of alloxan diabetic rat liver microsomes is lower than the activity in healthy animals (90). As expected, insulin administration caused an increase of enzyme levels to the normal range. Contrary to the results of the previous example, Huber et al. observed an increase of activity in response to glucagon

injection (90). However, this effect was transient in nature and was followed by depression of activity to below control levels. The mechanisms of these recently reported effects are yet to be elucidated.

Finally, it has been suggested that norepinephrine enhances HMG CoA reductase activity (91). Within twelve hours after norepinephrine injection cholesterol biosynthesis had doubled, as assayed in rat liver tissue slices. This increase was not a direct effect since addition of norepinephrine to a tissue slice incubation had no effect. Treatment with puromycin abolished this increase but did not inhibit conversion of labeled mevalonic acid to cholesterol. On this basis stimulation of enzyme synthesis was suggested as the mechanism of the norepinephrine effect.

Very recent work has shown that addition of 3',5'-cyclic AMP to rat liver microsomes, liver cells, and 5,000 x g liver supernatant lowers the incorporation of acetate and acetyl CoA into cholesterol but does not alter the incorporation of mevalonic acid (92). Similarly, addition of ATP and Mg^{++} to rat liver microsome preparations was shown to lower reductase activity. However, if the microsomal pellet was washed three times with buffer the ability of ATP and magnesium ion to inhibit the reductase was lost. This indicated that some soluble agent with inhibitory capabilities was present. On investigation of the nature of this agent it was found that addition of 100,000 x g

supernatant to washed microsomes restored the ability of ATP and magnesium ion to inhibit the enzyme. The inhibiting factor was found to be protein in nature since the reductase activity was also decreased by preincubation with material precipitated from 100,000 x g supernatant by ammonium sulfate. These experiments demonstrate the presence in rat liver cytosol of a protein, perhaps a kinase, which inhibits HMG CoA reductase activity. A possible mechanism for this inhibition may be an alteration of the conformation of the reductase by interaction with this protein material.

The previously discussed diurnal rhythm of cholesterol biosynthesis is caused by a diurnal rhythm of HMG CoA reductase (63, 78). This cyclic variation of enzyme level has been intensively studied but there remain several aspects which are not clearly defined or agreed upon. Maximum reductase levels occur at approximately midnight while the minimum occurs close to noon (93) and the difference in activity between these two points is five to tenfold. Study of the fine structure of the rhythm shows that, in fact, there are two maxima occurring at midnight and 1:45 AM (94). There are several possible explanations for this pair of maxima. A labile enzyme degrading system could appear for short periods destroying a portion of the increased levels of reductase present (78). It is also possible that there exists a metabolically programed limit to enzyme levels and when this limit is exceeded cellular

controls act to decrease enzyme synthesis (78). If this is true then it is reasonable to assume that both peaks are due to the same rise of enzyme synthesis. The decline of activity between these maxima would then be caused by a control overshoot and when the enzyme has reached low enough levels for the control system to stop functioning enzyme levels would begin to rise. It should be noted that these plausable explanations are speculative and their confirmation or disproval awaits further experimentation.

Fasting is known to depress cholesterol biosynthesis and therefore might be expected to alter the diurnal rhythm. Hamprecht et al. (95) showed that in fasted rats the rhythm persists but at a much lower amplitude than in fed controls. This has been interpreted as evidence that the rhythm is not a response to eating habits of the animals.

Several investigators have probed the mechanism and causative factors associated with the diurnal rhythm of this enzyme. One possible cause for the rhythm is changes in the rate of enzyme synthesis, degradation, or both. Measurement of formation and destruction constants for the reductase at various times during the cycle show that the breakdown constant changes very little but the formation constant increases seven to tenfold during the diurnal rise over that observed during diurnal fall (96). It therefore appears that the rhythm is due to increased enzyme synthesis during the rise with almost constant degradation

rates throughout the cycle. Other investigators have also implicated protein synthesis as the major rhythm controlling factor. Under the assumption that amino acid incorporation was a valid measure of protein synthesis, H^3-leucine incorporation into the enzyme has been studied (63, 97). Variation of radioactivity in the isolated enzyme accurately reflected the rise and fall of activity throughout the cycle. Increase of label incorporation precedes the cyclic enzyme rises by four to six hours and this may be the time necessary for protein synthesis to be reflected in enzyme levels. Higgins et al. (97) claim that these data provide unequivocal evidence that the diurnal enzyme variation is due to an increased rate of enzyme synthesis for six hours during cyclic rise followed by complete cessation of enzyme synthesis for fifteen hours during the cyclic decline.

Additional evidence for the role of enzyme synthesis was provided by the work of Edwards and Gould (93). Following injection of rats with cycloheximide, HMG CoA reductase activity always declined. If a degradatory protein were the actual cause of the rhythm, cycloheximide administration would have inhibited its synthesis and reductase levels would have declined slower than normal. This was not observed and enzyme synthesis was once again implicated. Shapiro and Rodwell's experiments are in complete disagreement with this hypothesis (98). They found that cycloheximide treatment caused a complete block

of the normal diurnal rise of reductase levels; however, enzyme levels remained elevated after cycloheximide treatment, indicating that the diurnal fall had also been blocked. These experiments are evidence that the diurnal rise is due to enzyme synthesis but that the fall is caused by synthesis of a degradatory protein rather than by cessation of reductase synthesis.

The relationship of feeding habits and light-dark cycles to the diurnal rhythm of this enzyme have been investigated. When the light-dark cycle under which experimental animals were housed was shifted by twelve hours the diurnal enzyme rhythm also exhibited a twelve hour shift within seven days (99), maintaining peak activity in the dark phase. During this same period food consumption was also shifted into the new dark phase, obscuring whether light and dark cycles or feeding habits were important factors. Similarly, regardless of the magnitude of the shift in light-dark cycles, maximum enzyme levels were always observed very close to six hours after the onset of darkness (93), however, since rats normally feed during darkness, food consumption always shifted also. Strong evidence for effects of food consumption was provided by experiments where feeding was restricted to the period 8:30 AM to 10:30 AM (96). Under these conditions enzyme levels rose rapidly and peaked at approximately noon. Rats maintained in continuous light for nine days exhibited a diurnal rhythm of normal magnitude but the peak enzyme

levels occurred slightly later than normal and minimum levels were slightly higher than control (100). Similarly, rats kept in continuous darkness for nine days showed normal rhythm where peak enzyme levels occurred slightly earlier than normal. Examination of the rhythm in continuous light conditions is the experiment suggested by Hickman et al. (60), and preservation of the rhythm under these conditions excludes corticosterone as a regulator of these cyclic variations. Finally, superior cervical ganglionectomy did not abolish reductase rhythm (100), indicating lack of involvement of the pineal gland.

Adrenalectomized rats have been shown to maintain normal diurnal rhythm of the reductase (101). This means that enzyme variations are not controlled by corticosterone, which is produced in the adrenal cortex, supporting the above results with continuous lighting, or by catecholamines, which are produced in the adrenal medulla. Considering the rate limiting nature of the reductase, this observation is contradictory to the experiments of Hickman et al. (60) who found that adrenalectomy abolished the diurnal rhythm of cholesterol biosynthesis but that the rate of biosynthesis was maintained at high levels. Clearly, an unknown factor appears to be related to both rhythms. Thus, in view of the contradictory nature of much of the evidence concerning diurnal variations, it appears that food consumption is a primary controlling factor but that other factors may also be involved.

Another aspect of the involvement of HMG CoA reductase with cholesterol synthesis and regulation is presented by hepatoma tissue. Numerous investigators have shown that cholesterol biosynthesis in several hepatomas does not respond to fasting and cholesterol feeding as do normal cells (63, 78, 102-106). This apparent loss of regulation has been observed in several types of hepatomas in rat (102, 104, 105), mouse (102, 103, 106), and man (104). Early investigations of this phenomenon were done by Siperstein and Fagan (102) who found that rat and mouse hepatomas synthesize cholesterol and that the site of dietary cholesterol effects, HMG CoA reductase, was indeed present and functioning. Their experiments showed that the absence of cholesterol biosynthesis regulation by dietary cholesterol was not caused by the rapid growth of tumor tissue, lack of HMG CoA reductase, or loss of the ability of tumor tissue to synthesize cholesterol; rather, it was concluded that the loss of regulation was due to malfunctioning regulatory mechanisms. Later studies of kinetic properties of HMG CoA reductase isolated from both tumor cells and normal liver cells demonstrated that the tumor enzyme was not measurably different from the normal enzyme (103). It therefore appeared that failure of regulation in hepatomas was due to the inability of dietary cholesterol to reach the site where it acts or to a defect in the regulatory system itself. In addition to the lack of control by dietary cholesterol or fasting, tumor tissue

exhibited no diurnal variations of cholesterol biosynthesis (107).

Attempts to experimentally define a defective regulatory system in these hepatomas may not be necessary since some investigators suggest the true defect to be inability of dietary cholesterol to enter hepatoma cells (63, 78, 106, 108, 109). Using mouse liver slices and mouse liver homogenates, Sabine (108) studied the effects of bile acids on hepatomas 5123C and BW7756 and found that cholesterol biosynthesis was not affected in these hepatomas by removal of bile acids. However, *in vitro* addition of bile acids to cell free hepatoma preparations caused inhibition of tumor cholesterol biosynthesis as effectively as normal cholesterol synthesis. On the other hand, addition of bile acids to whole cells exhibited inhibition in normal liver but not in hepatoma cells. The inescapable conclusion is that hepatomas do not exhibit regulation of cholesterol biosynthesis because the regulating agent is not able to enter the cells.

Finally, using label uptake experiments, Harry *et al.* (109) observed that administration of H^3-cholesterol to rats was followed by uptake of radioactivity into normal liver cells but not into Morris hepatoma 7787 cells. This provided clear evidence that the lack of cholesterol regulation by tumor cells was due to the inability of cholesterol to enter the cell, not to a regulatory system defect.

The above discussion shows that cholesterol, its

biosynthesis, and HMG CoA reductase play important roles in atherosclerotic diseases. In at least one case, familial hypercholesterolemia, HMG CoA reductase has been demonstrated to be the most important, if not causative, disease factor (110). Familial hypercholesterolemia is an autosomal dominant trait which the heterozygote usually carries for 30 to 60 years in an asymptomatic condition. Homozygotes for this gene develop (110): 1) extremely high plasma cholesterol levels, often exceeding 800 mg per 100 ml, 2) cutaneous planar xanthomas in early life, 3) coronary, cerebral, and peripheral occlusive vascular diseases in childhood. These conditions are associated with accumulation of cholesterol in atheromatous plaques. Death usually occurs in these individuals as a result of myocardial infarction often prior to the age of 30.

Goldstein and Brown (110) have recently studied HMG CoA reductase obtained from fibroblasts of homozygotes for familial hypercholesterolemia. Normal human fibroblasts maintained on fetal calf serum exhibited low levels of reductase activity. If the medium was replaced with human lipoprotein deficient plasma, reductase activity increased. When human low density lipoprotein was added, enzyme activity declined in a manner which was both time and concentration dependent. This observation is reminiscent of certain conditions which have regulatory effects on this enzyme. Under the same experimental conditions HMG CoA reductase from human homozygote fibroblasts

exhibited sixty times higher activity than normal human cells when maintained on fetal calf serum. Replacement of the calf serum with human lipoprotein deficient plasma or human low density lipoprotein was accompanied by no change in enzyme activity. This lack of regulation was shown to be unrelated to added materials since incubation of low density lipoprotein, derived from normal and homozygote subjects, with normal fibroblasts was able to depress enzyme activity. These experiments are a clear demonstration of defective HMG CoA reductase regulation being integrally related to a hypercholesterolemic disease with atherosclerotic complications.

Because kinetic properties of the reductase from normal and homozygote are nearly identical the genetic determinant for the enzyme itself does not appear to contain the defect. Further, a genetic alteration would cause changes of enzyme structure which would be expressed as changes of enzyme activity, not regulation. It was concluded that the genetic mutation of familial hypercholesterolemia causes a defect in the regulation of HMG CoA reductase activity by lipoproteins (110). It is possible that this could occur by mutation of a gene which normally specifies the elaboration of a protein which is critical to enzyme regulation. This regulatory defect results in the extremely high rate of cholesterol biosynthesis exhibited by the homozygote. Thus, the ability to inhibit cholesterol biosynthesis, preferably at the reduction of

HMG CoA to mevalonic acid with no other metabolic changes, would provide an effective means of treatment of familial hypercholesterolemia. Such an ability does not exist at this time.

D. Hypocholesterolemic Agents

Treatment of atherosclerotic diseases is confronted with a unique quandary. The problem arises from two considerations: first, the great difficulty of proving a causal relationship between hyperlipidemia and hypercholesterolemia and the diseases and second, the multifaceted nature of the several factors which evidently act together to produce these diseases. Thus, it is not possible to positively state that lowering of serum cholesterol and lipid levels will in fact aid in the prevention of atherosclerosis. This is emphasized by a statement from the American Heart Association Study Group, "Primary Prevention of the Atherosclerotic Diseases" when referring to drug treatment of atherosclerosis: "What is yet to be determined is whether biochemical action of these or similar drugs will exert any favorable effect on the cause of the atherosclerotic diseases and whether long-term continued use of these substances produces significant deleterious effects" (117). However, in spite of this uncertainty it is generally accepted that even though lowering of blood cholesterol and lipid levels may not provide an absolute

cure for atherosclerosis, it certainly cannot be harmful and might even be beneficial. Therefore, an important means of approaching atherosclerosis therapy is by attempting to lower blood levels of these substances.

Several methods are known for lowering cholesterol levels. Dietary measures can be designed which limit the consumption of cholesterol and other lipid substances although the magnitude of lowering obtained with these programs is often small. Enhancement of cholesterol excretion, metabolism, or both is a second method. Like dietary measures this program does not generally produce dramatic results. Furthermore, consumption of the large quantities of polymeric resins necessary to achieve these effects often acts as a negative incentive for such a therapeutic regimen. Finally, the most extensively explored method of lowering cholesterol levels is by inhibition of its biosynthesis. Several agents have been developed for this purpose and varying degrees of success have been achieved. A discussion of the choice of the inhibition site and the design of these agents will be found in a later section. Although a vast number of agents which reduce serum cholesterol levels have been synthesized, only a few of the more important examples will be considered here.

The initial approach to treatment of hypercholesterolemia and hyperlipidemia is dietary (25, 111). An early method of dietary management consisted of addition

of sitosterol to the diet. The rationale for this treatment was that sitosterol is not absorbed from the intestines but does form single crystals with cholesterol from which neither component can be physically separated. Pollak (112) found that feeding rabbits varying quantities of sitosterol along with enough cholesterol to induce atherosclerosis resulted in the lowering of blood cholesterol levels. This was a graded response; the higher daily sitosterol doses caused greater depression of cholesterol levels. In addition, sitosterol feeding protected against atherosclerosis which normally developed in cholesterol fed rabbits. Extension of these studies to humans afforded essentially identical results (113). In these experiments it was noted that the cholesterol lowering effects were greater for high initial blood levels than for low initial levels. One drawback of this method is that very high consumption of sitosterol (up to 10 grams per day) is required.

Dietary management of hypercholesterolemia can also be accomplished by adoption of a modified diet. These diets are usually designed to reduce obesity and to depress blood lipids as much as possible. A diet designed for moderate reduction of blood cholesterol levels eliminates butter fats and vegetable oils, replaces meat with fish, and employs skim milk and polyunsaturated fats. This diet has been shown to yield a 17% lowering of serum cholesterol levels (114). Similarly, strict cholesterol lowering

diets completely eliminate butter and margarine fats, drastically reduce meat consumption, and use high protein vegatables such as peas and beans. Strict observation of this diet reduces serum cholesterol levels 29% (114).

Many methods of dietary lowering of blood lipid levels replace saturated fats with unsaturated ones. The lipid lowering effects of unsaturated fats are not caused by enhanced fecal sterol excretion or redistribution of lipids to other body pools. Several investigators believe that these effects are due to changes in plasma lipoprotein compositions caused by unsaturated fats taking the place of saturated fats. In light of the established lipid lowering properties of unsaturated fats it has been proposed that these fats are incorporated into low density lipoprotein where, because of their unsaturation, they occupy more area than the corresponding saturated fats (115, 116). This may cause conformational changes of the lipoprotein resulting in fewer lipid molecules being accomodated by the protein portion of low density lipoprotein than when saturated fatty acids are incorporated. The overall result is that the total lipid content of lipoprotein is lowered. Although this hypothesis has not been rigorously proved, it is consistent with the finding that in unsaturated fat diets lipid-protein ratios showed less lipid to be present in low density lipoprotein than in saturated fat diets (115).

A thought provoking caution against abuse of

polyunsaturated fat diets has been pointed out by Mead (111) who expressed concern about the uncontrolled autooxidation of these fats. Autooxidation is the spontaneous oxidation of a compound by molecular oxygen. This process is usually very slow; however, unsaturated fatty acids are known to undergo autooxidation very readily in a manner similar to that shown in Figure 8. The initiator of this

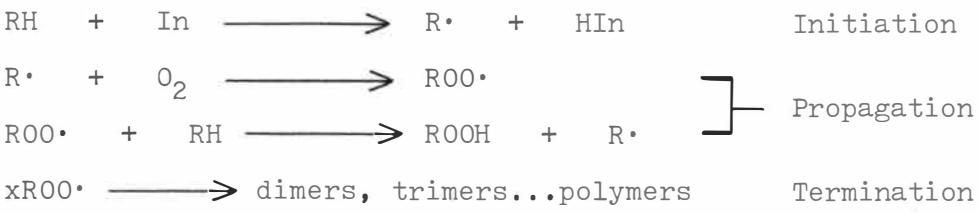


Figure 8: Possible sequences of free radical autooxidation reactions, after Mead (111). In = initiator.

process can be a free radical or a metal. It has been pointed out that peroxidized fats are known to be toxic materials and can form in biological tissues (111). Thus, drastically increasing the quantity of dietary polyunsaturated fats may be accompanied by a corresponding increase in opportunities for these lipid peroxide and free radical reactions to take place. If true, the result would be possible increases of tissue damage in excess of that which normally occurs. To this caution was added the suggestion that polyunsaturated fat diets should be accompanied by adequate antioxidant supplementation, such

as approximately 30 mg of d-∝-tocopherol per day (111). A good source of these compounds is vegetable oils which contain not only polyunsaturated fats but also the anti-oxidants required in nature to prevent the natural poly-unsaturated compounds from decomposing.

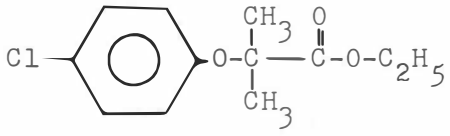
Perhaps the best example of reducing serum cholesterol levels by enhancement of metabolism and excretion is the use of cholestyramine. Cholestyramine is the chloride salt of a basic anion exchange resin composed of quater-nary ammonium groups attached to a polymeric styrene di-vinylbenzene skeleton with molecular weight in excess of one million. This resin lowers low density lipoproteins and therefore lowers plasma cholesterol levels, but does not affect very low density lipoproteins. By binding bile acids it prevents their reabsorption causing increased metabolism of cholesterol to bile acids. In addition, bile acids are required for intestinal absorption of cholesterol and therefore cholesterol reabsorption is also decreased.

In <u>in vitro</u> experiments cholestyramine was capable of removing large quantities of bile acids (118). Similarly, using cockerels and dogs as experimental animals, 1% cholestyramine in the diet prevented increases of blood cholesterol levels caused by cholesterol feeding. In addition, the resin is capable of lowering blood choles-terol levels of normocholesterolemic animals, indicating that the loss of cholesterol is greater than the

compensatory increases of biosynthesis. Similar results have been observed in man. Moore et al. (119) found that 12 grams of cholestyramine per day caused reduction of human serum cholesterol levels to 80% of pretreatment levels with a concurrent increase of fecal bile acid loss. The loss of bile acids continued in spite of the fact that serum cholesterol levels became constant at their low point, indicating that man responds with a compensatory increase of cholesterol synthesis, mobilization of cholesterol from extravascular depots, or both. However, since the low blood levels were maintained the compensatory mechanisms were not sufficient to return them to pretreatment levels.

The major drawbacks of cholestyramine therapy include the large daily quantities of drug required for positive results to be observed. A more serious complication is the anion exchange nature of cholestyramine. Since it is a basic resin it is capable of binding other drugs which are sufficiently acidic. It has been shown to bind chlorothiazide, phenylbutazone, phenobarbital, and tetracycline (154). This nonselectivity for bile acids could seriously compromise concurrent therapy.

Of the many compounds synthesized for the purpose of lowering serum cholesterol levels ethyl α-(4-chlorophenoxy) isobutyrate, also known as clofibrate, has received extensive study and application. This compound is a safe and effective agent for lowering serum cholesterol and triglyceride concentrations in man and has been



Clofibrate

demonstrated to be useful in all types of hyperlipidemia except the rare, fat induced hypertriglyceridemia (120). In addition to lowering human plasma cholesterol and triglyceride levels, clofibrate also enhances excretion of neutral sterols in the feces, increases mobilization of cholesterol from tissue depots (120), but does not appear to lower plasma triglycerides in rats or rabbits (121). Other workers have confirmed the finding that both cholesterol and triglyceride levels are lowered in man, although some patients do not respond at all (127). The most profound hypolipidemic effect is often seen in those individuals who had the highest blood lipid levels prior to treatment. Furthermore, Byers and Friedman also observed a 74% reduction of incorporation of H^3-acetate into cholesterol in the liver (121); however, the liver mass of treated animals was considerably greater than that of controls, causing cholesterol content per liver to increase. On the other hand, cholesterol content per gram of liver was indistinguishable for treated and control experiments. This led to the proposal that clofibrate reduces the rate

of hepatic cholesterol biosynthesis and thereby lowers the influx of cholesterol into the blood rather than increasing its efflux from the circulation. The increase of liver mass has also been studied by Platt and Thorp who found that when given 0.25% clofibrate in the diet the liver mass increased rapidly, remained constant for approximately sixty days, then slowly returned to normal by the termination of a two year regimen (122). For up to 29 days the dosage of clofibrate significantly increased protein content in rat liver; this effect appeared to precede the increase of liver mass and continued until total protein reached an equilibrium level. It is not known if the agent caused an increase of synthesis of all liver proteins or of a specific protein.

It was initially thought that clofibrate acted by potentiation of the metabolic effects of adrenal steroids and was therefore claimed to have maximum efficiency when used in combination with androsterone (126). This combination, known as Atromid, gained widespread clinical acceptance. Other investigators (123, 124) administered clofibrate to large numbers of human subjects for extended periods both with and without androsterone. Under both dosage regimens effects on serum proteins, side effects, and toxic effects were minimal. Both regimens were found to be equally effective in ability to lower elevated serum cholesterol levels, although 15-25% of the patients did not exhibit adequate or sustained reduction of either cholesterol or

triglycerides. The therapeutic effects were so similar to each other that it was concluded that the addition of androsterone to clofibrate was a therapeutically useless tactic (123).

The only major side effect of this drug is its interaction with anticoagulant agents (124, 125). Concurrent clofibrate and anticoagulant therapy requires reduction of the anticoagulant dose by 33-50% (125) in order to maintain optimum anticoagulant activity. No correlation was observed between hypolipidemic effects which appeared within two weeks and the alteration of coagulation properties which became noticeable within four weeks.

Many investigators have studied the mechanism of action of clofibrate and a large body of often contradictory evidence has resulted. It was initially thought that clofibrate was bound to serum albumin and other blood proteins at the same sites which normally bind L-thyroxine (122, 128). In order to bind at these sites the drug displaced thyroxine which was then taken up by liver cells. This hypothesis provided no function for the drug other than physical displacement of thyroxine, and the effects associated with clofibrate were actually thought to be due to normal hepatic response to the thyroid hormone. Support for this proposal was provided by the work of Chang et al. (128) who studied protein binding in vitro. In man the vast majority of thyroxine is normally bound to prealbumin, albumin, and thyroxine binding α-globulin.

Addition of 6 mM clofibrate to these binding proteins lowered the amount of thyroxine bound to prealbumin although higher concentrations were necessary to displace thyroxine from albumin and no displacement from thyroxine binding α-globulin was observed (128). Similar results were obtained in the dog. These experiments suggest that the drug acts by displacing the bound thyroid hormone, but do not exclude other possible mechanisms.

Best and Duncan (129) observed that adrenalectomized and gonadectomized rats responded to this agent with normal reductions of serum cholesterol levels. This experiment showed that clofibrate did not act by enhancing the action of endogenous steroid hormones. If these animals were made hypothyroid with thiouracil clofibrate caused no hypolipidemic effects. Similarly, thyroidectomized animals exhibited only a small reduction of serum cholesterol concentration in response to the drug. This work lent further support to the thyroid hormone displacement mechanism of action. On the other hand, administration of the drug to humans with complete primary myxedema resulted in lowered blood cholesterol and triglyceride concentrations (127) indicating that in man displacement of thyroxine may not be an important mechanism of action.

Early indications that clofibrate actually inhibits cholesterol biosynthesis resulted from in vitro experiments with cell free homogenates of bovine aorta (130). In this system the drug inhibited the conversion of labeled

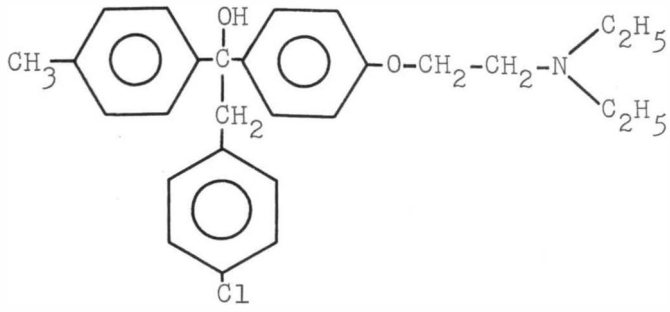
mevalonic acid to nonsaponifiable material. Support for the suggestion that inhibition of cholesterol biosynthesis was involved came from the experiments of Avoy et al. (131). Using a rat liver system these investigators showed that clofibrate drastically lowered the incorporation of labeled acetate into digitonin precipitable sterols, but when labeled mevalonic acid was employed as the precursor incorporation was not significantly inhibited. Similarly, in vivo experiments also showed that inhibition did not take place late in cholesterol biosynthesis. It was therefore concluded that clofibrate acted by inhibiting mevalonic acid synthesis from acetate but did not inhibit fatty acid or ketone body synthesis. Results of this type have also been reported by Sodhi et al. (132) using human Type II and Type IV hyperlipidemias. It should be noted that it is in this portion of cholesterol biosynthesis that the important regulatory reaction mediated by HMG CoA reductase is located.

The recent, definitive experiments of White (133) provided proof that a mechanism of action of clofibrate is indeed inhibition of cholesterol biosynthesis. At 0.3% in the diet it had no effect on food consumption or weight gain but consistently reduced rat plasma cholesterol levels by a mean of 35%. Using rat liver slices and cell free homogenates it was demonstrated that incorporation of radioactive acetate into cholesterol was decreased, but incorporation of labeled mevalonic acid was not changed. This

was in complete agreement with previous results (131). Furthermore, the incorporation of radioactivity into carbon dioxide and fatty acids was not altered by the drug and acetoacetate formation was increased in treated animals. This narrowed the location of inhibition to being between acetoacetyl CoA and mevalonic acid. Finally, clofibrate feeding was associated with no alteration of HMG CoA condensing enzyme but did cause decreased incorporation of radioactive HMG CoA into mevalonic acid. Thus, the inescapable conclusion (133) is that clofibrate acts, at least partially, by inhibiting hepatic microsomal HMG CoA reductase, the most appropriate site of action for maximum inhibitory efficiency. Although it was shown that the drug also interferes with the soluble enzyme system which activates acetate to acetyl CoA this is a secondary mechanism of action.

The recent work of Witiak et al. (155) emphasizes the fact that clofibrate has several modes of action. These investigators found that treatment of rats with clofibrate caused a significant increase of hepatic microsomal cytochrome P-450. This was interpreted (155) as an indication that another mode of action of clofibrate is increased catabolism of cholesterol due to the enhancement of hepatic cytochrome P-450.

Triparanol or 1-[p-(β-diethylaminoethoxy)phenyl]-1-(p-tolyl)-2-(p-chlorophenyl) ethanol (MER-29) is an agent which was initially thought to be a promising inhibitor of



Triparanol

cholesterol biosynthesis. Although it does inhibit the biosynthesis and reduce blood cholesterol levels, this compound is an excellent example of the disadvantages which accompany inhibition late in the biosynthetic sequence. Much of the promise of triparanol was due to early reports of its apparent ability to reduce total sterol synthesis. It was found that after feeding the compound to rats, incorporation of radioactive acetate into digitonin precipitable material was essentially unchanged with respect to untreated animals (134). However, when cholesterol was separated from the digitonin precipitate and purified it was discovered that the conversion of labeled substrate to cholesterol had been almost totally inhibited.

Further examination of the total digitonin precipitable material provided results applicable to the mechanism of action of triparanol. As previously reported, separation of cholesterol as its dibromide accounted for very small

amounts of total radioactivity; the majority stayed in the remaining digitonide. Investigation of the noncholesterol precipitate demonstrated that most of the labeled acetate had been incorporated into 24-dehydrocholesterol (desmosterol) (135). These experiments provided evidence that the reaction inhibited by triparanol was the reduction of 24-dehydrocholesterol to cholesterol and that desmosterol is a biosynthetic precursor of cholesterol. Further support for this mechanism was provided by the finding of very large amounts of desmosterol in the serum and liver of triparanol treated subjects, and undetectable amounts in control animals (136). Thus, triparanol treatment was associated with accumulation of desmosterol, and even though cholesterol synthesis was inhibited, very little reduction of the total sterol levels took place. Final proof of the biosynthetic relationship between desmosterol and cholesterol was made by Steinberg and Avigan (137) who conclusively demonstrated the precursor nature of desmosterol both in vitro and in vivo.

The high levels of desmosterol found in triparanol treated subjects is a serious drawback to the use of this agent to accomplish reduction of serum cholesterol levels because of the possibility that desmosterol itself may contribute to the atherosclerotic process. By experiments with rabbits, Avigan and Steinberg (138) were able to show that dietary desmosterol was indeed incorporated into atherosclerotic lesions. Examination of the grossly

normal aorta and lesions of desmosterol fed and triparanol treated rats showed that the ratio of desmosterol to cholesterol in these tissues was the same as the ratio of these sterols in the serum. Therefore, it appeared that triparanol inhibition of cholesterol biosynthesis was accompanied by at least partial replacement of cholesterol in lesions by desmosterol. From these studies it can be concluded that if desmosterol is present in the circulation it will be incorporated into developing atherosclerotic plaques at a rate which is proportional to its concentration in the serum. In view of these data, use of triparanol as a hypocholesterolemic agent would depend upon desmosterol being deposited in plaques at a slower rate than cholesterol. In humans treated with the drug for two to nine months desmosterol has been found in both uninvolved aorta tissue and in plaque, while no evidence of it could be detected in these tissues of untreated patients (139). This accumulation took place over a period of months while an entire plaque requires decades for development. This suggests a more rapid deposition of desmosterol than of cholesterol.

Because of the atherogenic properties of desmosterol and its rapid accumulation in lesions, the use of triparanol is not a rational method of treatment for atherosclerotic diseases. This compound clearly illustrates the major disadvantage of inhibiting cholesterol biosynthesis at one of the reactions close to the end product. The

result is the accumulation of cholesterol precursors which have no metabolic purpose and for which there are no alternate metabolic pathways available. In the case of triparanol, an additional danger is present because the accumulated intermediate is an atherogenic compound.

Thyroid function and thyroid hormones have been shown to have an effect on the development of atherosclerosis; normal, healthy, or supplemented thyroid function appears to lend protection against these diseases (20). Hyperthyroid individuals often exhibit low concentrations of cholesterol in plasma. Hypocholesterolemia is associated with thyroxine administration partly because the hormone causes increased conversion of cholesterol to bile acids to a greater extent than it causes increased cholesterol biosynthesis (146). These and similar considerations have been the basis of attempts to treat hypercholesterolemia with thyroid hormones and their analogs. In general, the thyroid hormone L-thyroxine causes significant reductions of serum cholesterol levels (140). Other effects of this compound include a general increase of metabolic rate, increase of heart rate, and increased oxygen consumption. Even though the serum cholesterol lowering properties of L-thyroxine are desirable the concurrent increase of heart rate and oxygen consumption may precipitate an anginal attack in a patient suffering from coronary heart disease. Thus, the goal of using thyroid hormones and their analogs as hypocholesterolemic agents is to separate the general

metabolic stimulatory properties of the hormones from their cholesterol lowering ability.

The dextrorotatory stereoisomer of thyroxine has been found to possess a partial separation of these properties (141-143). Although L-thyroxine causes a greater reduction of serum cholesterol concentrations it is also almost 100 times more potent than the D-isomer in causing cardiac hypertrophy and increased heart rate (140). This may be due to the greater localization of L-thyroxine in cardiac tissue while dextrothyroxine is localized mainly in liver tissue (140, 144). Similarly, the D-isomer causes much less stimulation of the general metabolic rate than L-thyroxine. Studies of D-thyroxine in human myxedematous patients indicate that the separation of metabolic stimulation is so pronounced that serum cholesterol levels are lowered with no observable increase of the basal metabolic rate (141). Other human studies of longer duration report similar success. Patients with idiopathic hypercholesterolemia and cardiovascular diseases are able to tolerate this agent with no serious side effects. Perhaps most significantly, patients with angina pectoris exhibit cholesterol lowering with no deterioration of the electrocardiogram and no change in cardiac compensation (142). This is important because the increases of oxygen consumption and heart rate caused by L-thyroxine aggravate this condition. Finally, this study shows that those subjects with the highest pretreatment blood cholesterol levels exhibit the

greatest lowering effects.

Studies of the mechanism by which D-thyroxine acts have produced some interesting results. Early work by Rabinowitz et al. (143) showed that radioactive cholesterol administered to humans was cleared from the serum two to threefold faster by those treated with D-thyroxine than by untreated subjects. Further, the expired carbon dioxide containing radioactive label was considerably increased in treated patients. These data led to the conclusion that the observed reductions of serum cholesterol level may have been due to enhanced metabolic destruction of cholesterol.

Other experiments indicate that thyroid hormones may act as enzyme inhibitors rather than stimulators of cholesterol catabolism. When examined in an in vitro system, triiodothyronine was found to inhibit the incorporation of labeled acetate into cholesterol more efficiently than thyroxine or other iodinated tyrosine analogs (145). When labeled mevalonic acid was the substrate the thyroid hormones provided much less inhibition of incorporation of the label into cholesterol. The greater activity of triiodothyronine than thyroxine observed in this system has been explained by the indication that thyroxine was bound much more firmly to nonspecific proteins than was triiodothyronine. This implies that the inhibitory activity was in fact a function of the concentration of unbound hormone rather than due to structural influences of the compounds. The ability to inhibit cholesterol biosynthesis

before but not after mevalonic acid is a familiar pattern which has previously been the source of preliminary suggestions that HMG CoA reductase was involved.

The hypothesis that HMG CoA reductase is a possible site of action for thyroid hormones gained clear support from the work of Eskelson et al. (146). Thyroidectomized animals exhibited low levels of HMG CoA reductase which were increased by thyroxine administration resulting in enhanced cholesterol biosynthesis. It has also been shown that the addition of L-triiodothyronine and L-thyroxine to rat liver homogenates from rats with normal thyroid function decreased cholesterol biosynthesis (145). Using rat liver homogenates Eskelson and co-workers observed 50% inhibition of cholesterol biosynthesis from radioactive acetate by 10^{-4} M triiodothyronine but no inhibition when labeled mevalonic acid was the substrate. Similarly, D-thyroxine inhibited the conversion of labeled acetate to cholesterol but L-thyroxine did not (146). By using I^{131}-triiodothyronine and I^{131}-thyroxine it was also shown that the thyroid hormones were nonspecifically bound to microsomal protein. From these results it was concluded that the rate limiting reaction of cholesterol synthesis, catalyzed by HMG CoA reductase, is under thyroid hormone control and that this reaction is inhibited by triiodothyronine. The high concentrations of thyroid hormones which were needed to inhibit the reductase and the protein binding study suggest that these compounds actually inhibit

the enzyme only after the protein binding sites have become saturated.

These data make it tempting to postulate a dual function of thyroid hormones in cholesterol biosynthesis: very low hormone levels may control enzyme synthesis while higher concentrations directly inhibit the rate limiting step for the entire sequence of reactions. This provides an additional example of the importance of HMG CoA reductase and its central position in cholesterol biosynthesis.

An example of agents specifically designed to inhibit HMG CoA reductase is the azasteroids prepared by Counsell and co-workers (147-149). Under the belief that cholesterol acted as a true feedback inhibitor of cholesterol biosynthesis by inhibiting the reductase, it was reasoned that if cholesterol was bound to the enzyme by means of van der Waals forces and hydrogen bonding then this binding association could be strengthened by employing nitrogen isosteres of cholesterol as inhibitors of the enzyme (147). Several nitrogen isosteres of cholesterol were prepared for this purpose. Using the percent reduction of serum cholesterol in the rat as a test system the most effective inhibitors prepared were the 22,25-diaza analogs, 20α-(2-dialkylaminoethyl)aminopregn-5-en-3β-ol (147). Initial attempts to define the site and mode of action of these compounds yielded mixed results. Although 22,25-diazacholestanol (SC11952) was designed as, and would hopefully

act as, an inhibitor of HMG CoA reductase, in vitro incorporation of labeled acetate into cholesterol by rat liver homogenates was only equal to or lower than that of untreated animals (148). However, labeled squalene, lanosterol, and desmosterol all exceeded the corresponding levels in untreated animals and labeled cholesterol was considerably lowered with respect to controls. It was concluded that under in vitro conditions the aza analogs of cholesterol were capable of inhibiting the desired enzyme and little emphasis was placed on the build-up of the steroid intermediates.

The observed accumulation of squalene, lanosterol, and desmosterol indicated that the compounds inhibited cholesterol biosynthesis at at least one additional site late in the pathway. Reexamination of the inhibitory properties under in vivo conditions showed that the goal of inhibition of HMG CoA reductase was not achieved. Rather, 22,25-diaza cholestanol was conclusively shown to inhibit the conversion of desmosterol to cholesterol (149, 150) as did triparanol and therefore cause accumulation of desmosterol in rat liver and plasma. It was later shown that this compound is also capable of inhibiting the rat liver Δ^7 as well as the Δ^{24} reductases which are necessary for sterol biosynthesis; the latter enzyme catalyzes the conversion of desmosterol to cholesterol (152). High concentrations are required to inhibit the Δ^7 reductase and therefore no evidence of this action

is observed *in vivo*.

A more potent diazacholesterol derivative is the 20, 25-diaza analog which lowered serum cholesterol and phospholipid concentrations to a greater degree than did triparanol (153). In rats and in man this compound reduced serum cholesterol but not total sterol concentrations. As in the case of the 22,25-diaza derivative, subjects treated with this compound exhibited an increased level of desmosterol (152). Thus, while these analogs of cholesterol were rationally designed they are not useful hypocholesterolemic agents because, like triparanol, they caused accumulation of the potentially atherogenic cholesterol precursor desmosterol.

RESEARCH RATIONALE

The previous discussion showed that a high serum cholesterol level is clearly one of the several factors which contribute to atherosclerotic disease and its many complications. Because much confusion surrounds the exact role of cholesterol and its relationship to atherosclerosis it can only be assumed that lowering the serum cholesterol level is a profitable therapeutic approach to treatment of the disease. Regardless of the lack of rigorous proof, it is believed that the vast literature concerning this aspect of the disease suggests the validity of this central assumption. The goal of this work is to contribute to efforts designed to lower serum cholesterol levels in mammals by defining some structural aspects of a series of compounds designed to inhibit cholesterol biosynthesis.

Several methods are available for the reduction of serum cholesterol levels. The currently favored initial method is to reduce the quantity of ingested cholesterol by strict dietary management. This procedure affords only fair results and may be accompanied by considerable difficulty on the part of the patient. This problem is manifested in the inability or unwillingness of the patient to adhere to the required dietary restrictions.

Another approach is to increase the oxidation of cholesterol to bile acids and thereby reduce cholesterol

levels. Bile acids act as feedback regulators of the conversion of cholesterol to the bile acids. Cholestyramine, a polymeric ion exchange resin, decreases the reabsorption of the bile acids in the intestinal tract and therefore removes the regulator of cholesterol conversion to bile acids. This results in an increased oxidation of cholesterol and lowers serum cholesterol levels. A secondary mode of action of this resin is the indirect decrease of intestinal cholesterol absorption. As noted previously, because of their ion exchange properties these resins may also remove other medicinal agents from the gastrointestinal tract and therefore, they cannot be used with many therapeutic regimens. Finally, an extensively studied method for lowering serum cholesterol levels is to inhibit cholesterol biosynthesis. The inhibition of cholesterol biosynthesis is a delicate problem and requires careful consideration but can provide rewarding results.

Cholesterol biosynthesis from acetyl CoA is a complex process which consists of more than twenty known reactions. At first, it might appear reasonable that inhibition at any one of these steps would effectively lower the total quantity of cholesterol biosynthesized and therefore safely reduce serum levels. Unfortunately, this is not true and has been illustrated above, especially in the case of triparanol and the azasteroids.

The first step of cholesterol biosynthesis, conversion of acetyl CoA to acetoacetyl CoA, is also an important part

of fatty acid synthesis. Inhibition of this reaction would therefore inhibit fatty acid biosynthesis as well as cholesterol biosynthesis and produce undesirable results (147, 156-158). Similarly, interference with formation of acetyl CoA is undesirable since this compound is the well known entrance into the tricarboxylic acid cycle. The next step in the biosynthetic pathway consists of condensation of acetoacetyl CoA with acetyl CoA to produce HMG CoA. This reaction is the branching point in fatty acid and cholesterol biosyntheses from which ketone bodies are derived. For this reason the inhibition of HMG CoA formation might also inhibit the latter process and is therefore undesirable (156, 157). Thus, inhibition of any reaction in cholesterol biosynthesis up to and including formation of HMG CoA is not an acceptable approach to blocking the pathway, primarily because the additional metabolic processes for which these intermediates are required would also suffer inhibition.

Later steps in the biosynthetic sequence following the cyclization of squalene might appear to provide potential inhibition sites. Unlike earlier steps, the biosynthetic reactions following squalene formation are not reversible. For this reason, efficient blockade of one of these reactions would be expected to lead to accumulation of the corresponding intermediate. Since no alternate metabolic pathways exist for these compounds this accumulation would continue unhindered.

An excellent example of this problem is the hypocholesterolemic agent triparanol which inhibits reduction of 24-dehydrocholesterol (desmosterol) to cholesterol (135). Blood levels of desmosterol under normal conditions are not detectable; however, shortly after triparanol treatment desmosterol concentration rapidly increases and its accumulation is metabolically uninhibited. An additional danger associated with this example of inhibition late in cholesterol biosynthesis is the observation of Herndon and Siperstein (139) that desmosterol is incorporated into atherosclerotic plaque and that this deposition takes place at a more rapid rate than cholesterol deposition. It is reasonable that inhibition of any of the several reactions in the later stages of cholesterol biosynthesis would lead to similar results. Since these intermediates appear to have no function other than as cholesterol precursors and since this portion of the pathway does not contain any reversible reactions, the most likely method of disposition of these compounds is their accumulation. The total quantity of cholesterol synthesized would be lowered but the total sterol concentration would increase. Therefore, it is believed that the inhibition of the reactions between squalene and cholesterol does not provide a rational approach to lowering serum cholesterol levels. Similar views have been expressed by other investigators (133, 156, 157).

The above considerations severely limit but do not

preclude the choice of a biosynthetic reaction on which rational attempts to inhibit cholesterol biosynthesis may be focused. For the purposes of this work HMG CoA reductase, which mediates the reduction of HMG CoA to mevalonic acid, is the reaction which is believed to provide the most appropriate opportunity for effective inhibition of cholesterol biosynthesis. The properties which bestow this unique quality on this enzyme are many.

All biosynthetic processes take place at a rate which can be described and measured. For those processes which consist of a series of reactions leading to a final product one of the reactions will be the slowest, or rate limiting, step under a given set of conditions. In cholesterol biosynthesis the rate limiting step is mediated by HMG CoA reductase (66, 63). Because of this property, inhibition of the reductase will cause a greater proportional decrease in the total quantity of cholesterol synthesized than the same degree of inhibition at any other reaction in the entire pathway. Since this is a general kinetic characteristic of the biosynthetic pathway, alterations of the physiological condition should not interfere with the relative efficiency of inhibition.

Although the rate limiting nature of HMG CoA reductase is an important reason for its choice as the inhibition target, several other positive aspects enhance its desirability. It will be noted that the formation of acetoacetyl CoA and HMG CoA are reversible reactions. The

biosynthesis of acetoacetyl CoA is directly reversible, that is, both the formation and the cleavage of acetoacetyl CoA are catalyzed by the same enzyme. On the other hand, the biosynthesis of HMG CoA is indirectly reversible since the condensation and cleavage reactions are mediated by separate enzymes. However, the following step, reduction of HMG CoA to mevalonic acid, is the first irreversible reaction of cholesterol biosynthesis (62). In marked contrast to the later stages of the pathway where sterols are abundant, inhibition of HMG CoA reductase will cause no accumulation of intermediates by virtue of the reversibility of the formation of its immediate precursors. Any accumulation of HMG CoA in response to inhibition of HMG CoA reductase will be disposed of through ketone body synthesis. Similarly, acetoacetyl CoA accumulation will be disposed of through fatty acid synthesis. Thus, the irreversible reduction means that only cholesterol biosynthesis will be inhibited with no concurrent accumulation of intermediates. The importance of this reductase is emphasized by the fact that it is also the major site of physiological control of cholesterol biosynthesis under both normal and experimental conditions. This function of the enzyme is discussed in detail in the preceding section.

For these reasons HMG CoA reductase is believed to be the ideal site for efficient inhibition of cholesterol biosynthesis and was therefore chosen as the target enzyme

in the present work. Although this conclusion is in agreement with the work of other investigators (159, 160) this view is not unanimous.

Holmes and DiTullio (156) have pointed out that mevalonic acid is a precursor not only of cholesterol but also of coenzyme Q which is associated with electron transport. Inhibition of HMG CoA reductase was therefore claimed to be accompanied by inhibition of coenzyme Q biosynthesis. In order to avoid possible harmful effects these investigators suggested that the best site for inhibition of cholesterol biosynthesis lies between mevalonic acid and squalene (156). Closer examination of the reductase reveals these fears to be less serious than they originally appeared to be. Since cholesterol is a vital steroid from which many others are produced complete blockade of its synthesis is not desirable. Partial inhibition of HMG CoA reductase should provide the greatest possible decrease of total cholesterol synthesis while allowing some mevalonic acid to be synthesized. Partial, rather than complete, inhibition of the enzyme therefore makes the mevalonic acid formed available for both coenzyme Q and cholesterol biosyntheses. Serious blockade of electron transport would be rapidly expressed by metabolic deficiencies. No investigations specifically concerning any relationship between coenzyme Q or electron transport and the inhibition of HMG CoA reductase are available. However, in studies of the administration of clofibrate [an agent which acts, in

part, by inhibiting HMG CoA reductase (133)] to animals (122) and man (120) no indications of coenzyme Q deficiency have been reported. Therefore, it is believed that the reasoning of Holmes and DiTullio (156) does not completely describe the relationship of mevalonic acid to both coenzyme Q and cholesterol biosyntheses. Thus, HMG CoA remains the ideal site for the inhibition of cholesterol biosynthesis.

Popják (161) has expressed the opinion that the inhibition of HMG CoA reductase as a means of blocking cholesterol biosynthesis is a rational approach only on the basis of preliminary considerations. The major objection to this site of inhibition is one of selectivity. Popják argues that inhibition of HMG CoA reductase without concurrent inhibition of dehydrogenase enzymes seems to be "a remote possibility." (161). This assertion appears to have been made on the assumption that the active sites of these enzymes must be similar because of similar functions of the enzymes and therefore an inhibitor of one would also interfere with the normal functioning of the rest. It should be noted that this assumption is not necessarily true. Even though similar enzymes may mediate similar chemical conversions, the geometry of the active site is not solely dependent on the type of reaction taking place. The structure of the substrate in regions remote from the reaction site may be an important determinant of the structure of the enzyme active site since the substrate

must be accomodated. For this reason two enzymes which mediate very similar reactions could conceivably have very different active sites. An illustration of this is the striking specificity of HMG CoA reductase for HMG CoA as substrate (62); peripheral selectivity is seen in the observation that HMG-S-pantetheine is not reduced by the enzyme (76).

In presenting his objection to HMG CoA reductase as the ideal site for the inhibition of cholesterol biosynthesis Popják proposed the inhibition of prenyl transferase on the basis of alternate metabolic pathways for the allylic phosphate precursors of farnesyl pyrophosphate (161). Inhibition of prenyl transferase, which mediates farnesyl pyrophosphate formation, would result in accumulation of 3,3-dimethylallyl pyrophosphate. It was suggested that this compound would be hydrolyzed to the corresponding free alcohol, which should be a good substrate for hepatic alcohol dehydrogenases. The coenzyme A thioester of the resulting acid is known to be carboxylated to trans-3-methylglutaconyl CoA, which is reversibly hydrated to HMG CoA (Figure 9). Thus, the overall result of inhibiting prenyl transferase is the production of more precursor of the substrate for this enzyme.

It should be noted that if prenyl transferase is chosen as an inhibition site several advantages of HMG CoA reductase are lost. Specifically, the site no longer corresponds to the rate determining step of cholesterol

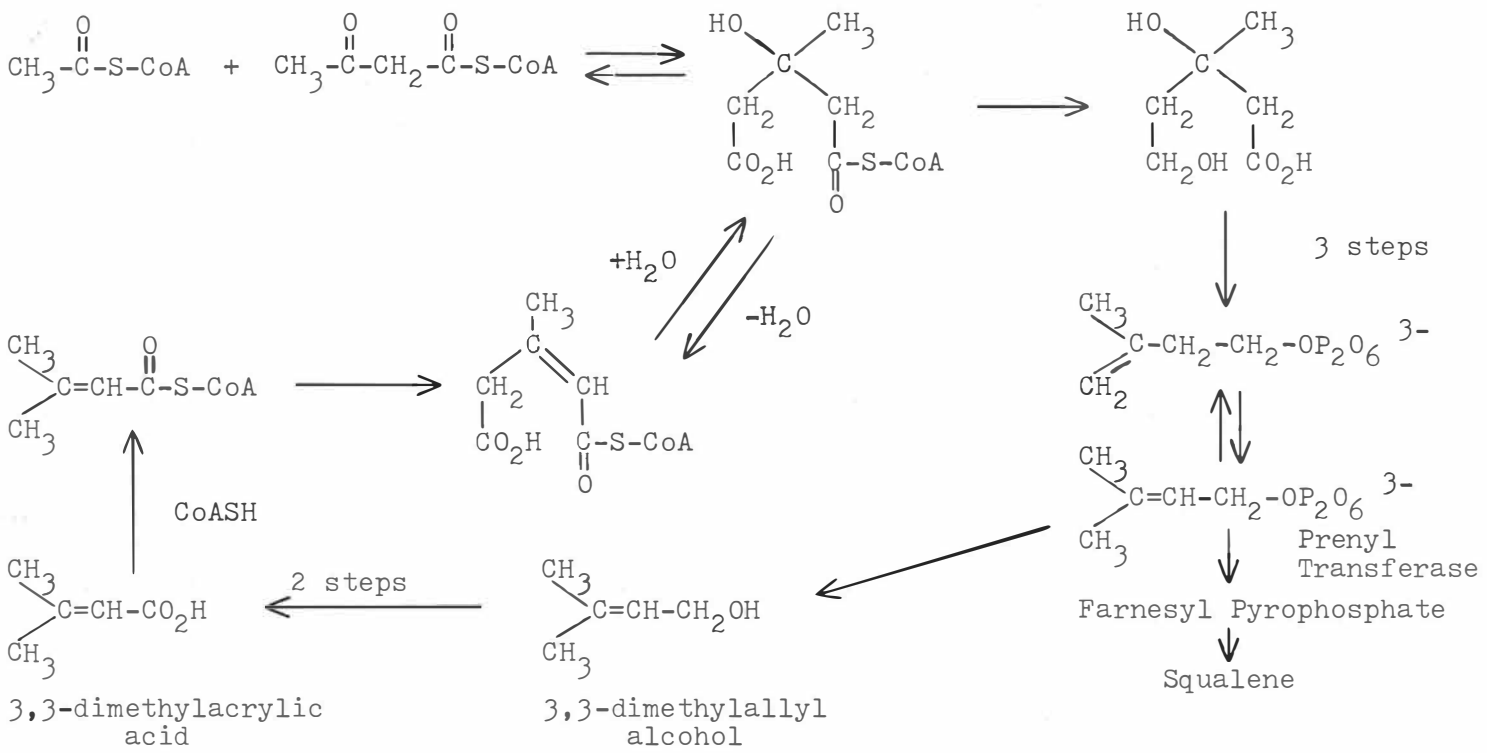


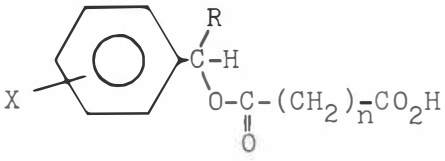
Figure 9: The alternate metabolic pathway proposed by Popják (161) for inhibition of prenyl transferase.

biosynthesis which is represented by HMG CoA reductase. Rather, inhibitory efficiency will be lost to the many slow reactions involved.

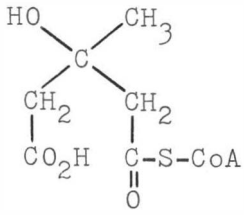
The above discussion shows that HMG CoA reductase is clearly the most appropriate site for the inhibition of cholesterol biosynthesis. For these reasons this enzyme has been chosen as the target enzyme for inhibitors designed and synthesized in the course of the present investigation.

Palazzo *et al.* (162) reported that a series of aryl-alkyl hydrogen succinates and glutarates I exhibited *in vivo* hypocholesterolemic activity in rats as well as the ability to inhibit *in vitro* cholesterol biosynthesis from labeled acetate. This investigation included the first report of the hypocholesterolemic and cholesterol biosynthesis inhibitory activities of the general class of 1-(4-biphenylyl)-*n*-alkyl hydrogen succinates and hydrogen glutarates. However, the effects of structural changes in these compounds on the hypocholesterolemic activity were not adequately explored by these workers. For this reason the only conclusion which can be drawn from this investigation is that esters I are capable of lowering serum cholesterol levels in the rat.

These results suggested to Boots *et al.* (158) that considerable information concerning the structural requirements for activity could be obtained by the incorporation of structural alterations into compounds similar to esters I. These investigators also noted the structural



I



II

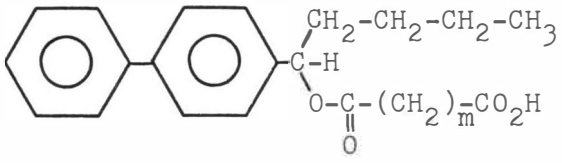
similarities between esters I and HMG CoA (II). Both contain free carboxyl and ester groups and for the series of hydrogen glutarates the distance between these two functional groups in the substrate and in the inhibitors can be identical. This led to the suggestion that these compounds might adopt similar conformations at their active sites and that these sites are located on HMG CoA reductase. In an extensive investigation of the inhibitory properties of some esters I in a yeast HMG CoA reductase system it was found (158) that as the alkyl group R was increased from hydrogen to n-hexyl in a series of compounds where X = p-phenyl, the activity reached a maximum when R was n-butyl or n-pentyl. This indicates that the R group of I contributes to binding by participating in hydrophobic interactions with the enzyme and that the hydrophobic binding area on the enzyme is large enough to accept an n-pentyl group.

A similar investigation was carried out to study the effects of substituents X on the binding of esters I (158).

While maintaining R as n-butyl, the substituent X was varied in such a manner that the electronic properties of the aromatic ring were known. Since these compounds exhibited no significant alteration of inhibitory activity, no electronic effects on inhibitor binding were observed. However, it was found that as the hydrophobic nature of the group X increased inhibition activity also increased; the most active compound contained X = p-phenyl. The excellent inhibition observed when the aromatic group was 2-fluorenyl showed that when X = p-phenyl the two aromatic rings were probably coplanar when bound to the enzyme. These experiments indicated that the aromatic rings also participate in binding by hydrophobic association with the target enzyme. Part of the site at which these compounds bind can therefore be envisioned as including a hydrophobic area which accomodates an n-alkyl group and a planar hydrophobic area at least large enough for bonding of a 4-biphenylyl moiety. The location of these areas relative to each other is a matter of surmise and is limited only by the conformations which inhibitors I can assume.

The acid-ester portion of these inhibitors also contains functional groups which may contribute to enzyme-inhibitor binding. Two such groups are the free carboxylic acid (-COOH) and the ester (-O-C(=O)-) function. In a study of possible distance requirements between these two functional groups during binding, Boots et al. (164) prepared a series of esters III in which the distance between the acid and

the ester groups was varied while the remainder of the molecule was maintained constant. Although a distance corresponding to one to four methylene groups between the ester and the acid moieties provided the most active inhibitors, no consistent increase or decrease of activity was observed throughout the series; only slight differences in activity were observed as the chain was lengthened. If the distance between the carboxylic acid group and the alcohol moiety of the ester was required to meet very demanding conditions in order to contribute to binding it would be anticipated that one member of this series would show a distinct maximum of inhibition while the other compounds would gradually increase to and/or decrease from maximum activity as the chain length was altered. However, the oxalate ester III (m = 0) exhibited very poor activity, indicating that when the distance between the ester and carboxylic acid groups was too small, proper binding of the carboxylic acid and alcohol moiety of the ester could not take place. These experiments lead to the conclusion that the carboxylic acid group does contribute



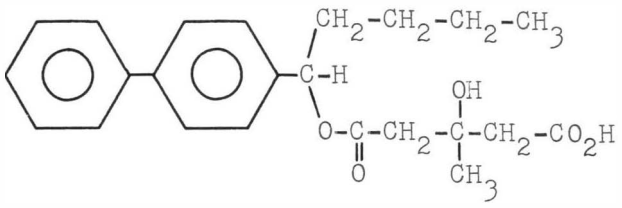
III (m = 0-6)

to binding and that as the chain length between the carboxylic acid and the ester group increased, the chain became sufficiently flexible to allow the carboxylic acid group and the alcohol moiety of the ester to reach their respective binding points.

Another potential binding point of inhibitors I is the ester group. By replacing each atom of this functional group the several possible modes of binding of which it is capable may be individually examined. Using succinate III (m = 2) as a reference inhibitor Boots et al. (164) prepared ester analogs for this purpose. Replacement of the carbonyl group of the ester with a methylene group afforded an ether which exhibited activity nearly identical to that of the reference ester. Similarly, replacement of both the carbonyl group and the ether oxygen of the ester with a pair of methylene groups caused a slight decrease in the inhibitory activity. These experiments suggest that the ester group is not necessary for binding.

Finally, the previously noted structural similarities between these inhibitors and the enzyme substrate (158) caused Boots et al. (164) to prepare the direct substrate analog IV. This compound was found to be an extremely active inhibitor and exhibited an elevenfold increase of inhibition over the corresponding unsubstituted ester III (m = 3). This showed that the 3-hydroxy group participated in binding to the enzyme.

It should be emphasized that all of the structural



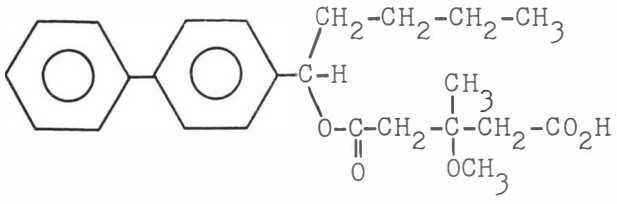
IV

influences on inhibition activity discussed above have been a measure of reversible interactions with some region of the enzyme surface. When an enzyme and its normal substrate interact, an enzyme-substrate complex (ES) forms. Similarly, in the case of the above inhibitors an enzyme-inhibitor complex (EI) is formed which decreases the ability of the enzyme to properly catalyze the reaction. In both cases, these complexes (ES and EI) are reversible in nature; that is, they may dissociate and form the unchanged enzyme and substrate or inhibitor species.

One of the immediate goals of the present work was to contribute to elucidation of some potential enzyme binding points of the above arylalkyl hydrogen succinates and hydrogen glutarates. The initial phase of this study was centered around the previously prepared substrate analog IV. Having suggested that the 3-hydroxy group of ester IV actively participates in binding to the enzyme, the question of the nature of this binding naturally followed. Two possibilities exist: A) the hydroxyl hydrogen could function as a hydrogen donor in hydrogen bonding with the enzyme

or B) the hydroxyl oxygen could act as a hydrogen acceptor in hydrogen bonding with the enzyme. These possible modes of binding are illustrated in Figure 10. It was therefore proposed to structurally modify inhibitor IV in such a manner to determine which, if either, of these binding modes was operating. The compound 1-(4-biphenylyl)-n-pentyl hydrogen 3-methyl-3-methoxyglutarate (V) was designed for this purpose.

Replacing the 3-hydroxyl hydrogen with a methyl group results in a compound which is not capable of acting as a hydrogen donor during hydrogen bonding (possibility A, above and Figure 10) but retains the ability to function as a hydrogen acceptor for hydrogen bonding (possibility B, above and Figure 10). If the first possibility accurately describes the contribution of the 3-hydroxy group, inhibitor V would be expected to exhibit decreased inhibition with respect to ester IV. Conversely, if the second possibility holds, little change of activity would be anticipated. The first phase of the present work consisted of synthesis of the 3-methoxy ester V which is described in the experimental section.



V

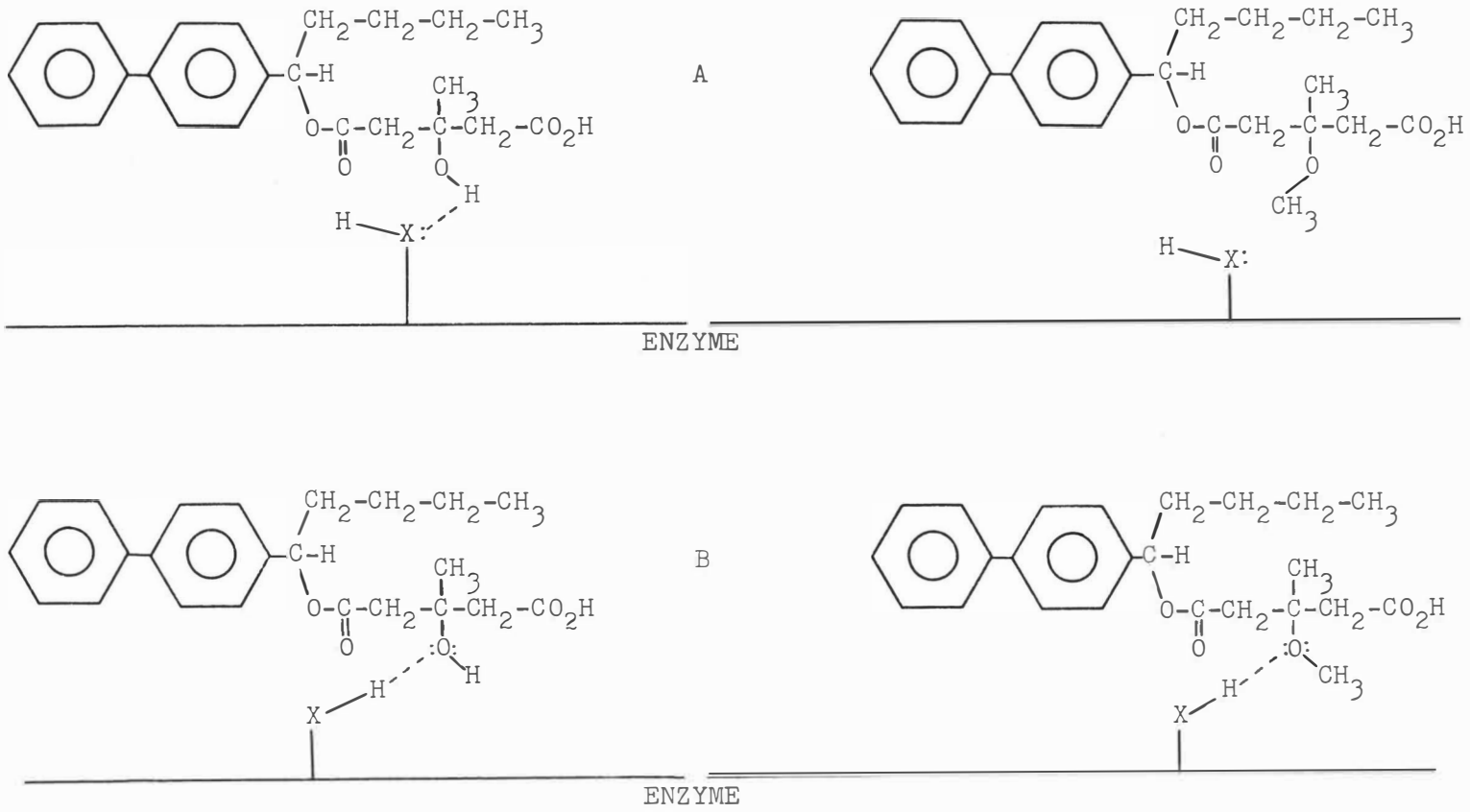
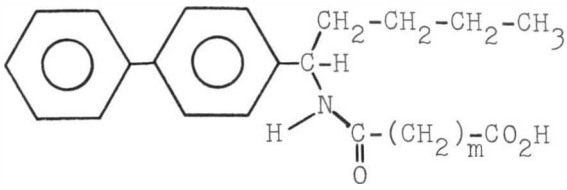


Figure 10: Possible modes of binding of the 3-hydroxy group of ester IV compared to the 3-methoxyl group of ester V. A, hydrogen bond donor; B, hydrogen bond acceptor.

The second phase of this work involving structural changes of possible binding points concerned the ester function. As discussed above, replacement of the carbonyl group and ether oxygen of the ester moiety with methylene groups did not significantly alter activity of III (m = 2). Under usual conditions the ester group can act as a hydrogen acceptor in hydrogen bonding by virtue of the free pairs of electrons on oxygen. As indicated above, this does not take place in this system.

Even though the above experiments show that the ester group is not necessary for binding of the inhibitor to the enzyme, it is possible that the enzyme surface contains unused functional groups capable of a binding contribution in the region where the ester moiety is normally located. The most likely character of such a group would be a hydrogen acceptor for hydrogen bonding since other similar possibilities have been excluded. To determine if such an enzyme surface exists the ester group of the inhibitor must be replaced with a functional group which maintains as much as possible the size, shape, and electronic properties of an ester but which also includes the ability to act as a hydrogen donor for hydrogen bonding. The closest approximation of these requirements is the amide. Amides VI and VII were prepared in an effort to probe this region of the enzyme and perhaps increase binding. Presence of a properly positioned enzymic hydrogen acceptor would be detected by an increase of inhibition by amides VI and



VI, m = 2
VII, m = 3

VII with respect to the corresponding ester, ether, and carbon analogs. Synthesis of these compounds is described in detail in the experimental section.

It has been noted that the previously prepared compounds and, in all probability, the compounds proposed for the present investigation are reversible inhibitors. In addition, it has been shown that succinate III exhibits noncompetitive kinetics and there is reason to suggest that a stable ternary complex of enzyme, substrate and inhibitor is formed (158). The kinetic behavior of these compounds indicates that they probably bind to HMG CoA reductase at some site other than the binding site for substrate and may therefore form an enzyme-substrate-inhibitor complex (ESI) which is not able to reduce HMG CoA to mevalonic acid. For this reason the mechanism of inhibition may be explained in a number of ways including alteration of enzyme conformation and interference with possible enzyme subunit interactions. Regardless of where on the enzyme these inhibitors bind, the reversible nature of the interaction means that inhibition will be temporary

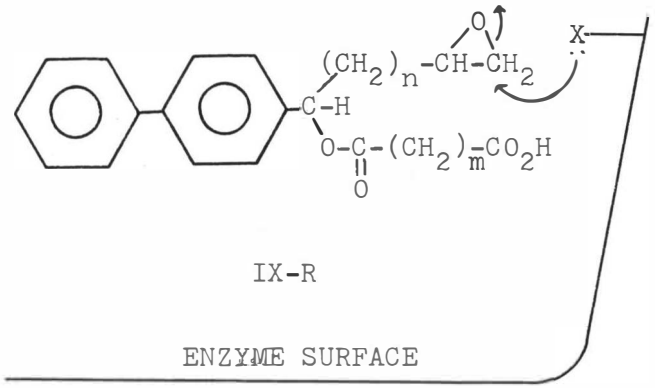
and limited by availability of the inhibitor to the biophase.

The elegant work of Baker (163) provided an alternative to the transient nature of reversible inhibitors. If an inhibitor can be designed which has the ability to form covalent bonds with the enzyme inhibition will become irreversible and more effective than reversible inhibition. The major portion of the present investigation consisted of the synthesis of structural analogs of esters I which contain a functional group which could be attacked by a nucleophile located on the surface of the enzyme. Such an attack would result in irreversible binding of the inhibitor to the enzyme surface.

The design aspect of incorporating into the inhibitors a functional group which is susceptible to nucleophilic attack is important. Many amino acids contain groups capable of acting as nucleophiles including the thiol group of cysteine, the hydroxyl group of serine and threonine, and the nitrogen of histidine. Since it is known that HMG CoA reductase requires free thiol groups for activity (62) an important nucleophile is certainly present on the enzyme surface and may attack the inhibitors. Even though these compounds presumably do not interact with the enzyme at its active site a covalently bound enzyme-inhibitor complex could do severe damage to catalytic efficiency since the attacking nucleophile may normally function as an aid to maintain the proper catalytic

conformation of the enzyme.

The compounds designed for this purpose were intended for examination of both reversible and irreversible inhibition properties. To maintain maximum reversible binding, as determined from previous investigations, the potential irreversible inhibitors contained the 4-biphenylyl group for its hydrophobic characteristics and the succinic or glutaric acid-ester groups. The epoxide function was chosen as the alkylating group. A major reason for the choice of the epoxide group as the alkylating moiety is the ease with which it is known to open when attacked by nucleophiles. A reasonable mechanism for opening the ring by an enzymatic nucleophile is shown in Figure 11. Additionally, this group is small and therefore would hopefully cause a minimum of steric hinderance problems with respect to approach of the inhibitor to the enzyme surface and subsequent reversible binding. The position at which the epoxide group was incorporated was the terminal point of the alkyl group R of esters I. In this manner the chain length can be increased and the area over which the epoxide can "search" for a nucleophile becomes considerable (Figure 12). In this manner it was hoped that an enzymic nucleophile at or near the inhibitor binding site could be found. The compounds proposed for this investigation include the alkenyl esters VIII which are reversible inhibitors and from which the potential irreversible inhibiting epoxyalkyl esters IX were prepared. The synthesis of



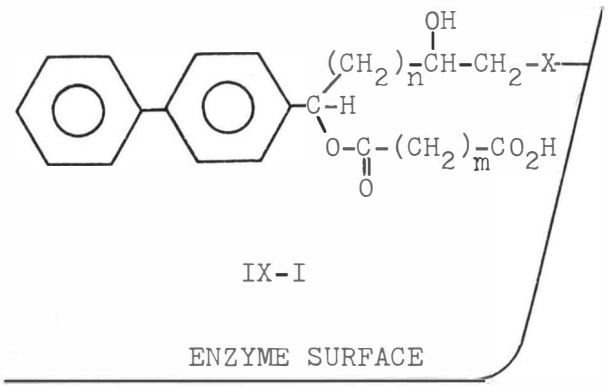


Figure 11: A mechanism by which irreversible inhibition might take place. The reversible enzyme-inhibitor comples (IX-R) forms, then the epoxide is attacked by an enzyme bound nucleophile (:X-) affording an irreversible covalently bound enzyme-inhibitor complex (IX-I).

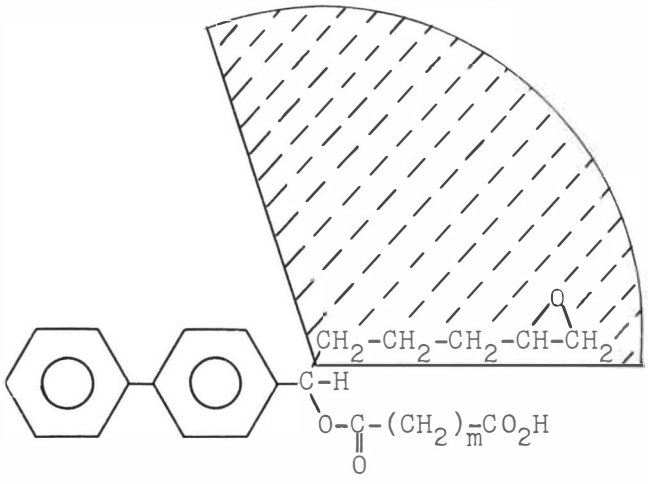
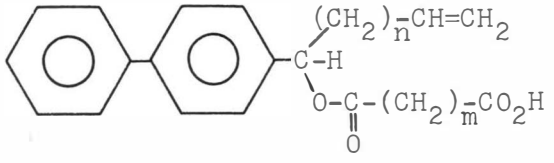
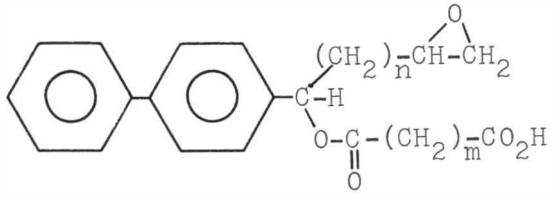


Figure 12: Under the assumption that the aryl and ester moieties always bind at the same location the shaded area represents the volume in which the epoxide group of inhibitors IXa-f may react with a nucleophile. The actual area over which the epoxide may alkylate is, of course, limited by the shape of the enzyme surface in the designated volume.



VIIIa-f, n = 1, 2, 3
m = 2, 3

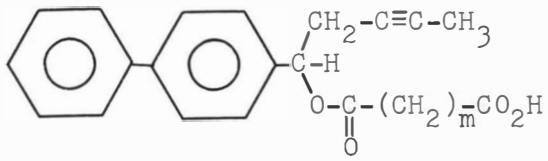


IXa-f, n = 1, 2, 3
m = 2, 3

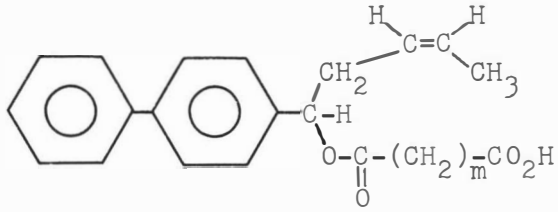
these compounds is described in the experimental section.

Finally, it was also desired to examine compounds of the type XI and XII containing the alkene function located at an internal position of the alkyl chain. This would allow stereochemical examination of the enzyme surface in the hydrophobic region where the alkyl group binds. From these compounds the corresponding internal epoxides could be prepared. Similarly, alkyne X, from which alkenes XI and XII could be synthesized, is structurally locked into linearity about the triple bond and could provide further information concerning the geometry of the enzyme in this binding region. As described in the discussion of the experimental section, synthetic difficulties precluded preparation of the unsaturated esters X-XII. However, it was possible to prepare alkynyl ester XIII which should provide similar information to that desired from X-XII

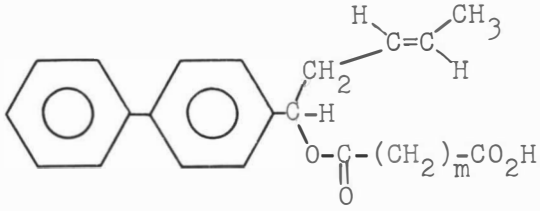
The most desirable system for evaluation of the above compounds is a pure preparation of the enzyme HMG CoA reductase. Such a preparation would assure that the alterations of inhibitory activity are accurate reflections of structural changes of the test compounds. In this manner interactions between the inhibitor and the enzyme could be studied without interference from effects due to absorption, distribution, or metabolism. However, a pure enzyme test system is not feasible because the enzyme is attached to the endoplasmic reticulum (65). Additionally, once it has



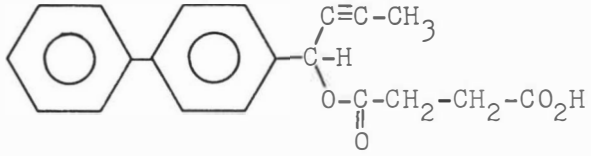
X



XI



XII



XIII

been removed from the endoplasmic reticulum, HMG CoA reductase is extremely fragile (69, 70) and may present handling problems if the solubilized preparation is used regularly for testing purposes.

These difficulties contributed to the decision to employ rat hepatic microsomal preparations of the target enzyme for inhibitor evaluation. A limitation to the use of microsomal preparations is the presence of other enzymes, some of unknown function. These enzymes represent possible sites of metabolic and absorption loss for the inhibitor. Even though the microsomal test system presents some disadvantages with respect to pure preparations it is not entirely without merit. Any inhibitor which proves to be especially promising will be examined in a whole animal system. Microsomes are a close approximation to the whole animal system because numerous enzymes are present in any whole animal system and in order to be effective a drug must be capable of evading possible deleterious effects of these enzymes. Therefore, the loss of the ideality inherent in pure enzyme systems is more than compensated for by a closer resemblance of microsomal systems to actual in vivo conditions.

EXPERIMENTAL

A. General

Reaction solvents were stored over the appropriate size molecular sieves (194) except dimethylformamide which was distilled from barium oxide prior to storage over molecular sieves and pyridine which was distilled from and stored over barium oxide. Tetrahydrofuran was stored over molecular sieves until the alkynyl systems were investigated. Following that time, this solvent was stored over lithium aluminum hydride and distilled immediately prior to use. Petroleum ether used for column chromatography was distilled prior to use, the 60-70° C fraction being collected. Removal of solvents under reduced pressure was done with a Buchler Flash Evaporator at water aspirator pressure and was followed by drying under a vacuum of less than one mm Hg.

Melting points are uncorrected and were determined on a Thomas Hoover melting point apparatus. Combustion analyses were performed with a Perkin-Elmer Model 240 Elemental Analyzer, or by Galbraith Laboratories, Knoxville, Tennessee. Infrared spectra were determined on a Perkin-Elmer Model 257 spectrophotometer either neat or as a solution in the indicated solvent. The strong, sharp band of polystyrene film at 1601.4 cm^{-1} was used as a reference marker for infrared spectra. Nuclear magnetic resonance

(nmr) spectra were measured on a Perkin-Elmer Model R-24 spectrometer or on a Varian A-60 spectrometer. Chemical shifts are given in units of parts per million (δ), in the indicated solvent, downfield from tetramethylsilane which was used as an internal standard. Liquid scintillation counting was performed on a Nuclear-Chicago Mark I Liquid Scintillation System and counting was corrected for efficiency by the external standard quench correction method.

All compounds are numbered with roman numerals. The S-benzylthiuronium salts are denoted by the number of the parent carboxylic acid followed by a capital S. For example, the salt of acid number VIIIc is denoted VIIIc-S.

B. Compounds Synthesized

4-Hydroxy-4-methyl-1,6-heptadiene (XIV). - The method of Tschesche and Machleidt (165) was used. To a three-necked flask fitted with a magnetic stirrer, pressure compensating dropping funnel, reflux condenser, and drying tube was added 19.05 g (0.78 g-atom) of magnesium turnings. The system was then flame-dried under a stream of dry nitrogen. When the system had cooled, 30 ml of dry ether, 30 ml of tetrahydrofuran and a crystal of iodine were added to the flask. To the magnesium mixture was added, in a dropwise manner over a period of 2-2.5 hours, a solution of 19.5 ml of ethyl acetate (0.198 mole), 57 ml of allyl bromide (0.66 mole), 75 ml of ether, and 225 ml of tetrahydrofuran. During the addition the reaction mixture was maintained at a gentle reflux and when the addition was complete the mixture was stirred at reflux for 2.5 hours. The mixture was poured onto an ice-water slurry and the aqueous phase was adjusted to pH 2 with 5% hydrochloric acid solution and extracted twice with ether. The combined organic phases were washed with water until the aqueous phase was pH 6, then with 5% sodium hydroxide solution, water, and then with a saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded an orange liquid which was distilled to give 24.8 g (89%) of XIV as a clear, colorless liquid bp 51° (15 mm), literature (165) bp 50-51° (12 mm).

The infrared spectrum showed λ_{max}^{neat} 3360 cm^{-1} (strong, O-H), 1645 cm^{-1} (strong, C=C). The nmr spectrum ($CDCl_3$) showed maxima for 2 protons as a multiplet at 5.60-6.37 ppm $(-CH=C<)_2$, 4 protons as a multiplet at 4.83-5.16 ppm $(>C=CH_2)_2$, 1 proton as a singlet at 2.38 ppm (R-O-H), 4 protons as a doublet at 2.17 ppm $(-CH_2-\overset{|}{C}=)_2$, and 3 protons as a singlet at 1.15 ppm (R_3C-CH_3).

4-Methoxy-4-methyl-1,6-heptadiene (XV). - The method of Anderson and Cree (166) was used. To a three-necked flask fitted with a gas inlet tube, drying tube, pressure compensating dropping funnel, and magnetic stirrer was added 3.7 g of sodium hydride as a 57% oil dispersion [2.1 g (0.088 mole) as sodium hydride] . The powder was washed three times by mixing with dry ether and allowed to settle, the supernatant being decanted after each wash, and dried by sweeping the vessel with dry nitrogen. Fifty ml of dry dimethylsulfoxide was then added with stirring to the system. To the stirred suspension was added at room temperature and in a dropwise manner a solution of 7.76 g (0.062 mole) of the previously prepared diene XIV in 25 ml of dry dimethylsulfoxide and the reaction mixture was stirred until gas evolution ceased. The flask was placed in an ice bath and to the system was added in a dropwise manner 26 g (0.18 mole) of methyl iodide. The mixture was stirred at room temperature for 18 hours, then the opaque orange solution was poured into ether and water. The organic phase was washed 7 times with water, followed by a

saturated solution of sodium chloride and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded a brown liquid which was distilled to give 5.64 g (65%) of XV as a clear, colorless liquid, bp 149-151° at atmospheric pressure. The infrared spectrum showed λ_{max}^{neat} 1645 cm^{-1} (strong, C=C). The nmr spectrum ($CDCl_3$) showed maxima for 2 protons as a multiplet at 5.50-6.16 ppm $(-CH=C\lesssim)_2$, 4 protons as a multiplet at 4.83-5.16 ppm $(\gtrsim C=CH_2)_2$, 3 protons as a singlet at 3.20 ppm $(R-OCH_3)$, 4 protons as a doublet at 2.25 ppm $(=\overset{|}{C}-CH_2-)_2$, and 3 protons as a singlet at 1.10 ppm $(R-CH_3)$.

Anal. Calcd. for $C_9H_{16}O$: C, 77.1; H, 11.5. Found: C, 77.2; H, 11.6.

3-Methyl-3-methoxyglutaric acid (XVI). - The method of Tschesche and Machleidt (165) was used. To a vacuum trap was added a solution of 7.36 g (0.053 mole) of previously prepared 1,6-heptadiene XV, 7.0 ml of glacial acetic acid, and 85 ml of methylene chloride. The system was put into a Dewar flask containing an acetone-dry ice slurry and cooled for 0.5 hour; then ozone (Welsbach ozone generator, Model T-408; 0.4 l./min, 0.562 kg/cm^2, 110 volt) was passed through the solution for 3.5 hours. The blue solution was removed from the acetone-dry ice slurry and allowed to warm to room temperature. To the resulting clear, colorless solution was added 50 ml of glacial acetic acid and the methylene chloride was removed under reduced pressure. To the residue was added 50 ml of glacial acetic acid, and

50 ml of 30% hydrogen peroxide; then the resulting solution was heated at reflux for 8 hours. Removal of the acetic acid under high vacuum afforded 7.39 g of a colorless oil which crystallized on standing under high vacuum. Recrystallization from ether-petroleum ether (60-70°) gave 7.08 g (76%) of XVI as a white solid, mp 78-80°. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 2500-2700 cm^{-1} (strong, broad, O-H), 1710 cm^{-1} (strong, C=O). The nmr spectrum (acetone-d_6) showed maxima for 2 protons as a broad singlet at 9.93 ppm $(-COOH)_2$, 4 protons as a singlet at 3.26 ppm $(-CH_2-)_2$, 3 protons as a singlet at 2.76 ppm $(R-OCH_3)$, and 3 protons as a singlet at 1.40 ppm $(R-CH_3)$. This material was used as an analytical sample with no further preparation.

Anal. Calcd. for $C_7H_{12}O_5$: C, 47.7; H, 6.9. Found: C, 47.7; H, 7.0.

1-(4-Biphenylyl)-pentan-1-ol (XVII). - The method of Palazzo et al. (162) as modified by S. G. Boots (personal communication) was used. To a three-necked flask fitted with a gas inlet tube, magnetic stirrer, reflux condenser, and pressure compensating dropping funnel was added 2.20 g (0.09 g-atom) of magnesium turnings. The system was then flame-dried under a stream of dry nitrogen. When the system had cooled a crystal of iodine was added to the mixture; then, with stirring and cooling in an ice bath, a solution of 7.2 g (0.052 mole) of n-butyl bromide in 70 ml of dry ether was added in a dropwise manner over a period of 1 hour while maintaining a gentle reflux. The

ice bath was removed and to the reaction mixture was added, in a dropwise manner with stirring, a solution of 9.2 g (0.05 mole) of 4-biphenylcarboxaldehyde, mp 52-55° (Aldrich), in 125 ml of dry ether while maintaining the system at a gentle reflux. The reaction mixture was stirred at reflux for 1 hour, cooled to room temperature and filtered to remove residual magnesium turnings. To the filtrate was added, in a slow dropwise manner with stirring, a saturated solution of ammonium chloride which had been adjusted to pH 7 with concentrated ammonium hydroxide solution. When no further precipitate formed the mixture was filtered, the solid was washed thoroughly with ether and the organic phase was dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 12.2 g of a yellow solid which was recrystallized from ether-petroleum ether (60-70°) to give 7.17 g (60%) of alcohol XVII as a white solid, mp 79-80°, literature (158) mp 78-79°. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3590 cm^{-1} (medium, free O-H), 3240 cm^{-1} (medium, broad, hydrogen bonded O-H). The nmr spectrum showed maxima for 9 aromatic protons at 7.20-7.70 ppm, 1 proton as a triplet at 4.57 ppm (R_3C-H), 1 proton as a singlet at 2.87 ppm (R-O-H), and 9 protons as a multiplet at 0.85-1.77 ppm (R-C_4H_9).

1-(4-Biphenylyl)-n-pentyl Hydrogen 3-Methyl-3-methoxyglutarate (V). - The ester was prepared by the procedure of Büchi et al. (167). To a solution of 880 mg (5.0 mmoles) of the previously described 3-methyl-3-methoxyglutaric acid

(XVI), mp 78-80°, in 25 ml of dry chloroform was added 1.20 g (5.0 mmoles) of alcohol XVII, mp 79-80°, and 1.59 g of N, N-dimethylformamide dineopentyl acetal (Aldrich); then the clear, yellow solution was stirred at room temperature for 68 hours. The solvent was removed under reduced pressure and the residue was dissolved in ether and washed with water followed by 5% sodium hydroxide solution. The alkaline aqueous phase was acidified to pH 1 with 5% hydrochloric acid and extracted with ether; then the organic phase was washed with water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 1.18 g (59%) of V as a viscous, clear brown oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1713 cm^{-1} (strong, C=O), 1740 cm^{-1} (strong, C=O). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a singlet at 10.0 ppm (-COOH), 9 aromatic protons at 7.00-7.79 ppm, 1 proton as a triplet at 5.71 ppm (R_3C-H), 3 protons as a singlet at 3.15 ppm ($-OCH_3$), 3 protons as a singlet at 2.72 ppm ($R-CH_3$), and 13 protons as multiplets at 0.67-2.00 ppm (methylene and $\underline{n}$-C_4H_9). The compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-n-pentyl Hydrogen 3-Methyl-3-methoxyglutarate (V-S). - The salt was prepared by the precedure of Donleavy (168) as modified by Boots et al. (158). To a solution of 306 mg (0.77 mmole) of the aforementioned hydrogen glutarate V in 15 ml of 95% ethanol was added 2.56 ml (0.77 mmole) of 0.3 N sodium

hydroxide solution followed by a solution of 1.1 g (5.0 mmoles) of S-benzyl-2-thiopseudourea hydrochloride (Eastman) in 8 ml of hot 95% ethanol. The resulting cloudy suspension was made up to 100 ml with distilled water and refrigerated for 12 hours. The mixture was filtered, the precipitate washed thoroughly with water and air dried. Two recrystallizations from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of the S-benzylthiuronium salt V-S as a white solid, mp 94.5-96°.

Anal. Calcd. for $C_{32}H_{40}N_2O_5S$: C, 68.0; H, 7.1; N, 5.0. Found: C, 68.2; H, 7.1; N, 5.1.

Preparation of the Jones Reagent. - The method of Bowden et al. (169) was used to prepare the reagent. To a solution of 28 g (0.28 mole) of chromium trioxide in 150 ml of distilled water was added slowly with stirring 25 ml of concentrated sulfuric acid. The resulting red solution was diluted to a total volume of 200 ml with distilled water to give a solution of 14 mmoles of the Jones reagent per 10 ml of solution.

1-(4-Biphenylyl)-pentane-1-one (XVIII). - The ketone was prepared by the procedure of Bowers et al. (170). To a solution of 1.02 g (4.2 mmoles) of alcohol XVII, mp 79-80°, in 10 ml of dry acetone was added 9 ml (12.6 mmoles) of Jones reagent in a dropwise manner with stirring and cooling in an ice bath to maintain the temperature below 10°. The resulting brown mixture was stirred for 0.5 hour at 0°. Ten ml of iso-propyl alcohol was added to destroy

excess Jones reagent, and the mixture was stirred for 1 hour at room temperature. The green reaction mixture was extracted with ether and water, and the combined organic phases were washed with a saturated solution of sodium chloride and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 1.11 g of ketone XVIII as white plates, mp 74-77°, which was recrystallized from absolute ethanol to give 802 mg (80%) of large white plates, mp 79.5-80.5°. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1669 cm^{-1} (strong, C=O). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.27-7.93 ppm, 2 protons as a triplet at 2.78 ppm ($-\overset{O}{\overset{\|}{C}}-CH_2-$), and 7 protons as a multiplet at 0.67-1.83 ppm ($-CH_2-CH_2-CH_3$).

Anal. Calcd. for $C_{17}H_{18}O$: C, 85.7; H, 7.6. Found: C, 85.6; H, 7.5.

1-(4-Biphenylyl)-pentane-1-methoxylimine (XIX). - The method of Feuer and Braunstein (171) was used. To a solution of 1.19 g (5.0 mmoles) of the previously described ketone XVIII, mp 79.5-80.5°, in 50 ml of absolute ethanol and 50 ml of pyridine (distilled from and stored over barium oxide) was added 431 mg (5.0 mmoles) of methoxylamine hydrochloride (Eastman). The solution was stirred at reflux for 24 hours, cooled to room temperature and then poured onto a slurry of ice and 5% hydrochloric acid. The mixture was extracted with ether and the organic phase was washed with water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the

solvent under reduced pressure afforded 1.21 g of viscous, yellow oil which solidified upon cooling and was recrystallized from ether-petroleum ether (60-70°) to give 610 mg (53%) of methoxylimine XIX as white needles, mp 43.5-45.5°. The infrared spectrum showed λ_{max}^{neat} 1610 cm^{-1} (weak, C=N-). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.23-7.90 ppm, 3 protons as a singlet at 3.97 ppm ($-OCH_3$), 2 protons as a triplet at 2.75 ppm ($-\overset{N}{\overset{\|}{C}}-CH_2-$), and 7 protons as multiplets at 0.67-1.67 ppm ($-CH_2-CH_2-CH_3$).

Anal. Calcd. for $C_{18}H_{21}NO$: C, 80.9; H, 7.9; N, 5.2. Found: C, 80.7; H, 8.1; N, 5.4.

1-(4-Biphenylyl)-1-aminopentane Hydrochloride (XX). - The method of Boots et al. (172) was modified. To a solution of 1.83 g (6.85 mmoles) of the aforementioned methoxylimine XIX, mp 43.5-45.5, in 25 ml of 1,2-dimethoxyethane (DME; distilled from lithium aluminum hydride) in a three-necked flask fitted with reflux condenser, pressure compensating dropping funnel, acetone bubbler, and magnetic stirrer was added 660 mg (17.2 mmoles) of sodium borohydride (Fisher). To the resulting mixture was added, in a dropwise manner, with stirring and cooling in an ice bath, 3.2 g (22.6 mmoles) of boron trifluoride etherate (Eastman) at a rate which controlled gas evolution and foaming. The reaction solution was stirred at room temperature for 1 hour, at reflux for 2 hours; then with stirring and cooling 3.0 ml of water was added in a dropwise manner followed by 15 ml of 5% hydrochloric acid solution. The resulting

solution was stirred at reflux for 1 hour then at room temperature for 14 hours. Removal of the DME under reduced pressure afforded a slurry which was adjusted to pH 12 with 5% sodium hydroxide solution and extracted with ether. The organic phase was washed with water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of most of the solvent under reduced pressure afforded a solution to which was added, with stirring at room temperature, 50 ml of ethereal hydrogen chloride [prepared (195) from addition of 5 ml of concentrated hydrochloric acid to 50 ml of ether followed by drying with 15 g of anhydrous magnesium sulfate and filtering]. Filtration of the resulting mixture afforded a white solid which on drying gave 1.60 g (85%) of amine hydrochloride XX as a white solid, mp 250-253°. The nmr spectrum (DMSO-d_6) showed maxima for 9 aromatic protons at 7.30-7.63 ppm, 3 protons as a singlet at 4.60 ppm ($-NH_3^+$) which was destroyed by the addition of sodium deuteroxide solution, and 9 protons as multiplets at 0.62-1.53 ppm ($\underline{n}$-C_4H_9). Recrystallization of this material from ethyl acetate afforded an analytical sample of XX as a white powder, mp 252-253.5°.

Anal. Calcd. for $C_{17}H_{22}NCl$: C, 74.0; H, 8.0; N, 5.1. Found: C, 74.4; H, 8.2; N, 5.4.

Succinic anhydride (XXI). - The procedure of Fieser and Martin (173) was used. To a flask fitted with a reflux condenser and gas trap leading to a solution of sodium

hydroxide was added 100 g (1.0 mole) of succinic acid, mp 182° (Fisher), and 315 ml (4.5 moles) of acetyl chloride. The reaction mixture was heated at reflux for 4 hours, cooled to room temperature and then refrigerated for 14 hours. The mixture was filtered and the solid was washed with ether. Concentration of the mother liquor afforded 13 g of white solid. The combined solids were dried under reduced pressure to give 79.4 g (79%) of succinic anhydride as white needles, mp 118.5-120.5°, literature (173) mp, 118-119°. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1785 cm^{-1} (strong, C=O), 1862 cm^{-1} (strong, C=O). The nmr spectrum (DMSO-d_6) showed a maximum as a singlet at 2.97 ppm ($-CH_2-CH_2-$).

N-[1-(4-Biphenylyl)-n-pentyl]-succinamic Acid (VI). - The method described by Vogel (174) was used. A solution of 0.50 g (1.82 mmoles) of the aforementioned amine hydrochloride XX, mp 250-253°, in 18 ml of ice-cold 5% sodium hydroxide solution was extracted with 50 ml of benzene. The organic phase was washed with water, a saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Filtration of the mixture afforded a solution of 435 mg (1.82 mmoles) of the desired 1-(4-biphenylyl)-1-aminopentane (XLIII) in dry benzene. To the solution was added 200 mg (2.0 mmoles) of the previously prepared succinic anhydride (XXI), mp 118.5-120.5°, and the resulting solution was stirred at room temperature for 3 hours, then extracted with 5% sodium hydroxide solution. The alkaline

aqueous phase was adjusted to pH 2 with ice-cold 5% hydrochloric acid solution, and the acidic material was extracted with ether. The organic phase was washed with water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure gave 573 mg of a white solid, mp 165-170°, which was recrystallized from ethyl acetate-petroleum ether (60-70°) to give 259 mg (40%) of succinamic acid VI as white needles, mp 176.5-178°. Thin layer chromatography of this material on neutral silica gel plates (Mallinkrodt) developed with 80% benzene-20% ethanol and visualized with both iodine vapor and bromcresol green showed the acid-amide to be pure within limits of detection with an R_f value of 0.65. The infrared spectrum showed λ_{max}^{KBr} 3250 cm^{-1} (strong, N-H), 1725 cm^{-1} (strong, C=O), and 1640 cm^{-1} (strong, C=O amide). The nmr spectrum (DMSO-d_6) showed maxima for 1 proton as a singlet at 8.44 ppm (-COOH), 1 proton as a singlet at 8.30 ppm (-CONH), 9 aromatic protons at 7.34-7.84 ppm, 4 protons as a singlet at 2.54 ppm (succinamic acid methylenes), and 9 protons as multiplets at 0.67-2.00 ppm (n-C_4H_9).

Anal. Calcd. for $C_{21}H_{25}NO_3$: C, 74.3; H, 7.4; N, 4.1. Found: C, 74.1; H, 7.4; N, 4.2.

N-[1-(4-Biphenylyl)-n-pentyl]-glutaramic Acid (VII). - The method described by Vogel (174) was used. To a solution of 1.03 g (4.3 mmoles) of 1-(4-biphenylyl)-1-aminopentane (XLIII) in 75 ml of dry benzene, prepared as

previously described for succinamic acid VI, was added 500 mg (4.3 mmoles) of glutaric anhydride, mp 52-56° (Aldrich). The resulting solution was stirred at room temperature for 72 hours and then extracted with 5% sodium hydroxide solution. The alkaline aqueous phase was adjusted to pH 1 with ice-cold 5% hydrochloric acid solution and the acidic material was extracted with ether. The organic phase was dried over anhydrous sodium sulfate and removal of the solvent at reduced pressure afforded 1.27 g of a white solid, mp 131.5-133.5°, which was recrystallized from ethanol-water to give 754 mg (50%) of glutaramic acid VII as a white solid, mp 136-137°. The infrared spectrum showed λ_{max}^{KBr} 3240 cm^{-1} (strong, N-H), 1705 cm^{-1} (strong, C=O), and 1630 cm^{-1} (strong, C=O amide). The nmr spectrum (DMSO-d_6) showed maxima for 1 proton as a singlet at 8.44 ppm (-COOH), 1 proton as a singlet at 8.30 ppm (-CONH), 9 aromatic protons at 7.34-7.75 ppm, 1 proton as a broad doublet at 4.92 ppm (R_3C-H), 6 protons as a multiplet at 1.58-2.42 ppm (glutaramic acid methylenes), and 9 protons as a multiplet at 0.67-1.50 ppm (n-C_4H_9).

Anal. Calcd. for $C_{22}H_{27}NO_3$: C, 74.8; H, 7.7; N, 4.0. Found: C, 74.6;H, 8.0; N, 3.9.

1-(4-Biphenylyl)-3-buten-1-ol (XXII). - The procedure of Tschesche and Machleidt (165) was used. To a three-necked flask fitted with a mechanical stirrer, reflux condenser, pressure compensating dropping funnel, and drying tube was added 0.97 g (0.04 g-atom) of magnesium turnings.

The system was flame-dried under a stream of dry nitrogen, and when it had cooled 10 ml of dry ether, 10 ml of dry tetrahydrofuran, and a small crystal of iodine were added to the flask. To the magnesium mixture was added in a dropwise manner over a period of 0.5 hour a solution of 3.46 ml (0.04 mole) of allyl bromide (Fisher), 3.64 g (0.02 mole) of 4-biphenylcarboxaldehyde (Aldrich), 25 ml of dry ether, and 75 ml of dry tetrahydrofuran. During the addition the reaction mixture was maintained at a gentle reflux and when the addition was complete the mixture was stirred at reflux for 1 hour. To the mixture was added in a slow, dropwise manner with cooling and stirring a saturated solution of ammonium chloride which had been adjusted to pH 7 with concentrated ammonium hydroxide solution. When no further precipitate formed the mixture was filtered and the white solid was washed with ether. The resulting solution was washed with a saturated sodium chloride solution and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 4.05 g of a light tan solid, mp 60.5-62°, which was recrystallized from hexane to give 3.09 g (69%) of butenyl alcohol XXII as white plates, mp 63.5-65°. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3590 cm^{-1} (medium, O-H), 1640 cm^{-1} (medium, $C{=}CH_2$). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.20-7.70 ppm, 1 proton as a multiplet at 5.48-6.18 ppm (-CH=), 2 protons as multiplets at 4.87-5.27 ppm ($=CH_2$), 1 proton as a triplet at 4.65 ppm (R_3C-H),

1 proton as a singlet at 2.68 ppm (O-H), and 2 protons as a triplet at 2.47 ppm ($-CH_2-$).

Anal. Calcd. for $C_{16}H_{16}O$: C, 85.7; H, 7.2. Found: C, 86.0; H, 7.2.

1-(4-Biphenylyl)-3-butenyl Hydrogen Succinate (VIIIa). The method of Steglich and Höffle (175) was modified. To a solution of 546 mg (2.44 mmoles) of the aforementioned butenyl alcohol XXII, mp 63.5-65°, in 10 ml of N,N-dimethylformamide (DMF), which had been distilled from barium oxide, was added 244 mg (2.44 mmoles) of succinic anhydride (XXI), mp 118.5-120.5°, and 297 mg (2.44 mmoles) of 4-dimethylaminopyridine, mp 108-110° (Aldrich). The resulting solution was stirred at room temperature for 72 hours and then poured into a slurry of ice and 5% hydrochloric acid and extracted with ether. The organic phase was washed with water, saturated sodium chloride solution and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 624 mg (79%) of hydrogen succinate VIIIa as a viscous, clear, yellow oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1715 cm^{-1} (strong, C=O), 1738 cm^{-1} (strong, C=O), and 1642 cm^{-1} (weak, C=C). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a singlet at 9.47 ppm (-COOH), 9 aromatic protons at 7.17-7.56 ppm, 2 protons as a multiplet at 5.34-5.90 ppm ($=CH_2$), and 6 protons as a singlet (succinate methylenes) superimposed on a triplet (butenyl methylene) at 2.55 ppm. This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-3-butenyl Hydrogen Succinate (VIIIa-S). - To a solution of 233 mg (0.72 mmoles) of hydrogen succinate VIIIa in 15 ml of 95% ethanol was added 2.56 ml (0.77 mmole) of 0.3 N sodium hydroxide solution and a solution of 1.1 g (5.0 mmoles) of S-benzyl-2-thiopseudourea hydrochloride (Eastman) in 8 ml of hot 95% ethanol. The volume was made up to a total of 100 ml with distilled water and the resulting opaque white mixture was cooled in an ice bath, filtered, and the precipitate was washed thoroughly with water. Two recrystallizations from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt VIIIa-S as a white solid, mp 143-144°.

Anal. Calcd. for $C_{28}H_{30}N_2O_4S \cdot \frac{1}{2}H_2O$: C, 67.3; H, 6.2; N, 5.6. Found: C, 67.4; H, 6.2; N, 5.6. After drying under a pressure of 1 mm Hg for 8.5 hours at 110° the one-half mole of water was successfully removed. The resulting elemental analyses were as follows:

Anal. Calcd. for $C_{28}H_{30}N_2O_4S$: C, 68.6; H, 6.2; N, 5.7. Found: C, 68.5; H, 6.1; N, 6.1.

1-(4-Biphenylyl)-3-butenyl Hydrogen Glutarate(VIIIb). - The method of Steglich and Höffle (175) was modified. To a solution of 896 mg (4.0 mmoles) of butenyl alcohol XXII, mp 63.5-65°, in 10 ml of N,N-dimethylformamide were added 456 mg (4.0 mmoles) of glutaric anhydride (Aldrich), mp 52-56°, and 488 mg (4.0 mmoles) of 4-dimethylaminopyridine (Aldrich), mp 108-110°. The resulting solution was stirred

at room temperature for 48 hours, then poured onto a slurry of ice and 5% hydrochloric acid and extracted with ether. The organic phase was extracted with a cold 5% sodium hydroxide solution; then the alkaline aqueous phase was adjusted to pH 2 with ice-cold 5% hydrochloric acid. The acidified material was extracted with ether and the organic phase was washed with water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 417 mg (26%) of hydrogen glutarate VIIIb as a viscous, light brown oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1730 cm^{-1} (strong, C=O), 1715 cm^{-1} (strong C=O), and 1645 cm^{-1} (weak, C=C). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a broad singlet at 9.20 ppm (-COOH), 9 aromatic protons at 7.17-7.60 ppm, 2 protons as multiplets at 5.37-5.94 ppm (R_3C-H and -CH=), 2 protons as a multiplet at 4.80-5.24 ppm ($=CH_2$), and 8 protons as multiplets at 1.17-2.74 ppm (glutarate and butenyl methylenes). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-3-butenyl Hydrogen Glutarate (VIIIb-S). - The salt was prepared by the method of Donleavy (168) as modified by Boots et al. (158) from the aforementioned hydrogen glutarate VIIIb in the same manner as previously described using 260 mg (0.77 mmole) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt VIIIb-S,

mp 146.5-147°.

Anal. Calcd. for $C_{29}H_{32}N_2O_4S$: C, 69.0; H, 6.4; N, 5.6. Found: C, 69.2; H, 6.5; N, 5.5.

1-(4-Biphenylyl)-3,4-epoxybutan-1-ol (XXV). - The method described by Fieser and Fieser (176) was used. To a solution of 3.36 g (15.0 mmoles) of the previously described butenyl alcohol XXII, mp 63.5-65°, in 25 ml of dry chloroform was added in a dropwise manner with stirring and cooling in an ice bath a solution of 5.06 g (25.0 mmoles) of 85% m-chloroperbenzoic acid (Eastman) in 100 ml of dry chloroform. The clear, colorless reaction solution was stirred at room temperature for 18 hours and then with 75 ml of a 10% solution of sodium sulfite until the starch-potassium iodide test was negative. The organic phase was washed with a 5% solution of sodium hydrogen carbonate, then with water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure gave 3.50 g (97%) of epoxyalcohol XXV as a brown oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3600 cm^{-1} (medium, free O-H), 3450 cm^{-1} (medium, broad, hydrogen bonded O-H), and 835 cm^{-1} (epoxide). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.27-7.66 ppm, 1 proton as a triplet at 4.84 ppm (R_3C-H), 1 proton as a singlet at 3.48 ppm (R-O-H), 3 protons as a multiplet at 2.32-3.22 ppm (-CH(O)-CH_2), and 2 protons as a triplet at 1.90 ppm (-CH_2-). Molecular distillation using a Hickman still provided an analytical

sample of this compound as a clear, colorless oil.

Anal. Calcd. for $C_{16}H_{16}O_2$: C, 80.0; H, 6.7. Found: C, 79.8; H, 6.5.

1-(4-Biphenylyl)-3,4-epoxybutyl Hydrogen Succinate (IXa). - The ester was prepared from the aforementioned epoxybutanol as previously described for the butenyl hydrogen succinate VIIIa to give 896 mg (57%) of epoxybutyl hydrogen succinate IXa as a viscous, yellow oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1740 cm^{-1} (strong, C=O), 1719 cm^{-1} (strong, C=O), and 840 cm^{-1} (strong, epoxide). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a singlet at 8.55 ppm (-COOH), 9 aromatic protons at 7.21-7.64 ppm, 1 proton as a triplet at 5.94 ppm (R_3C-H), 4 protons as a singlet at 2.60 ppm (succinate methylenes) superimposed upon 5 protons as a multiplet at 1.83-3.13 ppm ($-CH_2-\overset{O}{\overset{/\backslash}{CH-CH_2}}$). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-3,4-epoxybutyl Hydrogen Succinate (IXa-S). - The salt of the aforementioned epoxybutyl hydrogen succinate IXa was prepared in the previously described manner using 261 mg (0.77 mmole) of the hydrogen succinate. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt IXa-S as a light brown solid, mp 114-116°.

Anal. Calcd. for $C_{28}H_{30}N_2O_5S\cdot\frac{1}{2}H_2O$: C, 65.2; H, 6.0; N, 5.4. Found: C, 65.3; H, 6.0; N, 5.4.

1-(4-Biphenylyl)-3,4-epoxybutyl Hydrogen Glutarate (IXb). - The ester was prepared from the aforementioned epoxybutanol XXV as previously described for the butenyl hydrogen succinate VIIIa to give 325 mg (47%) of hydrogen glutarate IXb as a viscous, clear, yellow oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1735 cm^{-1} (strong, C=O), 1715 cm^{-1} (strong, C=O), and 835 cm^{-1} (strong, epoxide). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a broad singlet at 8.39 ppm (-COOH), 9 aromatic protons at 7.18-7.62 ppm, 1 proton as a triplet at 5.95 ppm (R_3C-H), and 8 protons as multiplets at 0.93-3.00 ppm (methylenes). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-3,4-epoxybutyl Hydrogen Glutarate (IXb-S). - An analytical sample of the S-benzylthiuronium salt of the aforementioned hydrogen glutarate was prepared as previously described using 259 mg (0.73 mmole) of the ester. Recrystallization of the crude salt twice from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt IXb-S as a white solid, mp 132-133°.

Anal. Calcd. for $C_{29}H_{32}N_2O_5S \cdot \frac{1}{2}H_2O$: C, 65.8; H, 6.2; N, 5.3. Found: C, 65.6; H, 6.2; N, 5.3.

1-(4-Biphenylyl)-4-penten-1-ol (XXIII). - To a three-necked flask fitted with a reflux condenser, drying tube, pressure compensating dropping funnel, and mechanical stirrer was added 486 mg (0.02 g-atom) of magnesium turnings

and the system was flame-dried under a stream of dry nitrogen. To the cooled system was added, in a dropwise manner with heating and stirring, a solution of 2.74 g (0.02 mole) of 4-bromo-1-butene (K&K Laboratories) in 50 ml of ether. To the resulting Grignard reagent was added a solution of 1.82 g (0.01 mole) of 4-biphenylcarboxaldehyde, mp 57-59° (Aldrich), in 50 ml of ether in a dropwise manner with stirring and heating at reflux over a period of 1.5 hours, followed by stirring at room temperature for 3 hours. To the reaction mixture was added in a slow, dropwise manner with stirring and cooling a saturated solution of ammonium chloride which had been adjusted to pH 7 with concentrated ammonium hydroxide solution. When no more precipitate formed the mixture was filtered, the solid was washed with ether, and the solution was dried over anhydrous sodium sulfate. Removal of the solvent at reduced pressure afforded 2.27 g (94%) of pentenyl alcohol XXIII as a white solid, mp 66.5-67.5°. Thin layer chromatography on silica gel (Eastman Chromotogram Sheet 6060) developed with 50% benzene-50% methanol and visualized with iodine vapor showed this material to be pure within the limits of detection. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3600 cm^{-1} (medium, free O-H), 3443 cm^{-1} (weak, broad, hydrogen bonded O-H), and 1641 cm^{-1} (medium, C=C). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.21-7.68 ppm, 1 proton as a multiplet at 5.53-6.10 ppm (-CH=), 2 protons as a multiplet at 4.81-5.20 ppm ($=CH_2$), 1 proton

as a triplet at 4.63 ppm (R_3C-H), 1 proton as a singlet at 2.50 ppm (O-H), and 4 protons as a multiplet at 1.67-2.34 ppm (-CH_2-CH_2-). Recrystallization of this compound from petroleum ether (60-70°) afforded an analytical specimen of alcohol XXIII as white needles, mp 68-68.5°.

Anal. Calcd. for $C_{17}H_{18}O$: C, 85.7; H, 7.6. Found: C, 85.6; H, 7.7.

1-(4-Biphenylyl)-4-pentenyl Hydrogen Succinate (VIIIc). The ester was prepared from the aforementioned pentenyl alcohol XXIII as previously described for the butenyl hydrogen glutarate VIIIb to give 1.05 g (57%) of hydrogen succinate VIIIc as a viscous yellow oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1718 cm^{-1} (strong, C=O), 1730 cm^{-1} (strong, C=O). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a singlet at 9.94 ppm (-COOH), 9 aromatic protons at 7.25-7.71 ppm, 2 protons as a broad multiplet at 5.50-6.09 ppm (R_3C-H and -CH=), 2 protons as a multiplet at 4.84-5.22 ppm ($=CH_2$), 4 protons as a singlet at 2.58 ppm (succinate methylenes), and 4 protons as a broad band centered at 2.04 ppm (butenyl methylenes). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-4-pentenyl Hydrogen Succinate (VIIIc-S). - The salt of the aforementioned hydrogen succinate VIIIc was prepared as previously described from 270 mg (0.37 mmole) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) gave an analytical sample of

S-benzylthiuronium salt VIIIc-S as a white solid, mp 118-119°.

Anal. Calcd. for $C_{29}H_{32}N_2O_4S$: C, 69.0; H, 6.4; N, 5.6. Found: C, 68.8; H, 6.4; N, 5.6.

1-(4-Biphenylyl)-4-pentenyl Hydrogen Glutarate (VIIId). The ester was prepared from the aforementioned pentenyl alcohol XXIII as previously described for the butenyl hydrogen glutarate VIIIb to give 735 mg (38%) of hydrogen glutarate VIIId as a viscous, yellow oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1712 cm^{-1} (strong, C=O), 1729 cm^{-1} (strong, C=O). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a singlet at 9.90 ppm (-COOH), 9 aromatic protons at 7.17-7.66 ppm, 2 protons as a broad band at 5.45-6.03 ppm (R_3C-H and -CH=), 2 protons as a multiplet at 4.78-5.19 ppm ($=CH_2$), and 10 protons as multiplets at 1.67-2.67 ppm (glutarate and butenyl methylenes). This compound was analytically characterized as described below.

S-Benzylthiuronium salt of 1-(4-Biphenylyl)-4-pentenyl Hydrogen Glutarate (VIIId-S). - The salt of the aforementioned hydrogen glutarate VIIId was prepared as previously described from 128 mg (0.36 mmole) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of the S-benzylthiuronium salt VIIId-S as a light brown solid, mp 122-123°.

Anal. Calcd. for $C_{30}H_{34}N_2O_4S \cdot \frac{1}{2}H_2O$: C, 68.3; H, 6.4; N, 5.3. Found: C, 68.4; H, 6.5; N, 5.4.

1-(4-Biphenylyl)-4,5-epoxypentan-1-ol (XXVI). - The method described by Fieser and Fieser (176) was used. To a flask fitted with a magnetic stirrer, pressure compensating dropping funnel, and drying tube was added a solution of 1.19 g (0.05 mole) of the previously prepared pentenyl alcohol XXIII, mp 66.5-67.5°, in 25 ml of dry chloroform. To the flask was added in a dropwise manner with stirring and cooling over a period of 1 hour a solution of 1.41 g (0.07 mole) of 85% m-chloroperbenzoic acid (Eastman) in 75 ml of dry chloroform. The reaction solution was stirred at room temperature for 20 hours, and then with 50 ml of a 10% solution of sodium sulfite until the starch-potassium iodide test was negative. The organic phase was washed with a 5% sodium hydrogen carbonate solution, water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent at reduced pressure afforded 1.23 g (96%) of epoxyalcohol XXVI as a brown oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3595 cm^{-1} (medium, free O-H), 3430 cm^{-1} (medium, broad, hydrogen bonded O-H), and 835 cm^{-1} (medium, epoxide). The nmr spectrum showed maxima for 9 aromatic protons at 7.24-7.67 ppm, 1 proton as a triplet at 4.66 ppm (R_3C-H), 1 proton as a singlet at 3.25 ppm (O-H), and 7 protons as multiplets at 1.17-2.79 ppm (epoxybutyl).

Several attempts were made to prepare an analytical sample of this compound. Molecular distillation with a Hickman still at temperatures up to 180° and pressure below

1 mm Hg afforded a clear, colorless oil which exhibited carbon analysis which was consistently approximately 1.6% below the theoretical value. Infrared and nmr spectra of this compound were satisfactory. In addition, the infrared and nmr spectra as well as elemental analysis for pentenyl alcohol XXIII, from which epoxyalcohol XXVI was synthesized, and for esters IXc and IXd which were synthesized from epoxyalcohol XXVI were all completely satisfactory. On this basis it was concluded that the desired epoxyalcohol XXVI had been obtained.

1-(4-Biphenylyl)-4,5-epoxypentyl Hydrogen Succinate (IXc). - The ester was prepared from the aforementioned epoxypentanol XXVI as previously described for the butenyl hydrogen glutarate VIIIb to give 924 mg (73%) of hydrogen succinate IXc as a viscous, yellow oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1720 cm^{-1} (strong, C=O), 1735 cm^{-1} (strong, C=O), and 835 cm^{-1} (medium, epoxide). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a singlet at 9.22 ppm (-COOH), 9 aromatic protons at 7.06-7.67 ppm, 1 proton as a broad band at 4.98 ppm (R_3C-H), 4 protons as a singlet at 2.62 ppm (succinate methylenes), and 7 protons as multiplets at 0.77-2.17 ppm (epoxybutyl). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-4,5-epoxypentyl Hydrogen Succinate (IXc-S). - The salt of the aforementioned hydrogen succinate was prepared as previously described from 272 mg (0.77 mmole) of the ester. Two

recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt IXc-S as a light brown solid, mp 97-99°.

Anal. Calcd. for $C_{29}H_{32}N_2O_5S \cdot \frac{1}{2}H_2O$: C, 65.8; H, 6.0; N, 5.3. Found: C, 65.7; H, 6.1; N, 5.4.

1-(4-Biphenylyl)-4,5-epoxypentyl Hydrogen Glutarate (IXd). - The ester was prepared from the aforementioned epoxypentanol XXVI as previously described for the butenyl hydrogen glutarate VIIIb to give 707 mg (42%) of hydrogen glutarate IXd as a clear, viscous yellow oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1730 cm^{-1} (strong, C=O), 1720 cm^{-1} (strong, C=O), and 835 cm^{-1} (weak, epoxide). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a singlet at 7.86 ppm (-COOH), 9 aromatic protons at 7.14-7.64 ppm, 1 proton as a broad band at 4.96 ppm (R_3C-H), and 10 protons as multiplets at 0.80-2.80 ppm (glutarate methylenes and epoxybutyl). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-4,5-epoxypentyl Hydrogen Glutarate (IXd-S). - The salt of the aforementioned hydrogen glutarate IXd was prepared as previously described using 176 mg (0.48 mmole) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt IXd-S as a tan solid, mp 115-117°.

Anal. Calcd. for $C_{30}H_{33}N_2O_5S \cdot \frac{1}{2}H_2O$: C, 66.3; H, 6.3;

N, 5.2. Found: C, 66.1; H, 6.4; N, 5.0.

1-(4-Biphenylyl)-5-hexen-1-ol (XXIV). - To a three-necked flask fitted with a reflux condenser, mechanical stirrer, drying tube, and pressure compensating dropping funnel was added 607 mg (0.025 g-atom) of magnesium turnings and the system was flame-dried under a stream of dry nitrogen. To the cooled system was added a small crystal of iodine and, in a dropwise manner with stirring over a period of 15 minutes, a solution of 2.98 g (0.02 mole) of 5-bromo-1-pentene (K&K Laboratories) in 50 ml of dry ether and the resulting mixture was stirred at reflux for 20 minutes. To the resulting Grignard reagent was added a solution of 3.09 g (0.017 mole) of 4-biphenylcarboxaldehyde, mp 52-55° (Aldrich), in 75 ml of dry ether and 15 ml of tetrahydrofuran in a dropwise manner over a period of 1 hour with stirring and heating at reflux, followed by stirring at room temperature for 2 hours. To the reaction mixture was added in a slow, dropwise manner with cooling and stirring a solution of saturated ammonium chloride which had been adjusted to pH 7 with concentrated ammonium hydroxide solution. When no more precipitate formed, the mixture was filtered, the solid was washed with ether, and the solution was dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure gave 3.01 g of a soft, brown solid which was recrystallized from petroleum ether (60-70°) to give 1.99 g (46%) of hexenyl alcohol XXIV as a white, fluffy solid, mp 54-57°. The infrared spectrum

showed $\lambda_{max}^{CHCl_3}$ 3599 cm^{-1} (medium, free O-H), 3435 cm^{-1} (weak, broad, hydrogen bonded O-H), and 1641 cm^{-1} (medium, C=C). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.16-7.67 ppm, 1 proton as a multiplet at 5.45 ppm (-CH=), 2 protons as a multiplet at 4.77-5.21 ppm ($=CH_2$), 1 proton as a triplet at 4.61 ppm (R_3C-H), 1 proton as a broad singlet at 3.02 ppm (O-H), and 6 protons as multiplets at 1.15-2.25 ppm ($-CH_2-CH_2-CH_2-$). Recrystallization of this compound from petroleum ether (60-70°) provided an analytical specimen, mp 55-58°.

Anal. Calcd. for $C_{18}H_{20}O$: C, 85.7; H, 8.0. Found: C, 85.5; H, 7.6.

1-(4-Biphenylyl)-5-hexenyl Hydrogen Succinate (VIIIe). The ester was prepared from the aforementioned 5-hexen-1-ol XXIV as previously described for the butenyl hydrogen glutarate VIIIb to give 612 mg (58%) of hydrogen succinate VIIIe as a beautiful, golden, viscous, clear oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1730 cm^{-1} (strong, C=O), 1720 cm^{-1} (strong, C=O). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a singlet at 8.55 ppm (-COOH), 9 aromatic protons at 7.18-7.74 ppm, 1 proton as a multiplet at 5.45-6.08 ppm (-CH=), 2 protons as a multiplet at 4.80-5.20 ppm ($=CH_2$), 1 proton at 4.65 ppm (R_3C-H), 4 protons as a singlet at 2.60 ppm (succinate methylenes), and 6 protons at 1.08-2.25 ppm ($-CH_2-CH_2-CH_2-$). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-5-hexenyl

Hydrogen Succinate (VIIIe-S). - The salt of the aforementioned ester VIIIe was prepared as previously described from 168 mg (0.48 mmole) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) provided an analytical sample of the S-benzylthiuronium salt VIIIe-S as a white solid, mp 125-127°.

Anal. Calcd. for $C_{30}H_{34}N_2O_4S \cdot 1H_2O$: C, 67.2; H, 6.3; N, 5.2. Found: C, 67.3; H, 6.5; N, 5.3.

1-(4-Biphenylyl)-5-hexenyl Hydrogen Glutarate (VIIIf). The ester was prepared by the method of Steglich and Höffle (175) from the aforementioned hexenyl alcohol XXIV as previously described for the butenyl hydrogen glutarate VIIIb. This afforded 457 mg (42%) of hydrogen glutarate VIIIf as a clear, viscous brown oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1730 cm^{-1} (strong, C=O), 1725 cm^{-1} (strong, C=O). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a broad singlet at 10.0 ppm (-COOH), 9 aromatic protons at 7.30-7.75 ppm, 2 protons as multiplets at 5.46-6.07 ppm (-CH= and R_3C-H), 2 protons as a multiplet at 4.67-5.20 ppm ($=CH_2$), and 12 protons as multiplets at 1.17-2.62 ppm ($-CH_2-CH_2-CH_2-$ of hexenyl and glutarate moieties). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-5-hexenyl Hydrogen Glutarate (VIIIf-S). - The salt of the previously described 5-hexenyl hydrogen glutarate VIIIf was prepared

as previously described using 174 mg (0.48 mmole) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt VIIIf-S as white plates, mp 100-101.5°.

Anal. Calcd. for $C_{31}H_{36}N_2O_4S$: C, 68.7; H, 6.6; N, 5.2. Found: C, 68.3; H, 6.5; N, 4.8.

1-(4-Biphenylyl)-5,6-epoxyhexane-1-ol (XXVII). - The method described by Fieser and Fieser (176) was employed. To a flask equipped with a pressure compensating dropping funnel, magnetic stirrer and drying tube was added a solution of 1.00 g (4.0 mmoles) of the previously described hexenyl alcohol XXIV, mp 54-57°, dissolved in 25 ml of dry chloroform. To the resulting solution was added in a dropwise manner with stirring and cooling over a period of one hour a solution of 1.21 g (6.0 mmoles) of 85% m-chloroperbenzoic acid (Eastman) dissolved in 75 ml of dry chloroform. On completion of the addition, the clear, colorless reaction solution was stirred at room temperature for 24 hours, then stirred at room temperature with 50 ml of 10% sodium sulfite solution until a negative starch-iodide test was observed. The organic phase was then washed with 5% sodium hydrogen carbonate solution, water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 989 mg (92%) of epoxyalcohol XXVII as a clear, light brown oil. The infrared spectrum

showed $\lambda_{max}^{CHCl_3}$ 3595 cm^{-1} (medium, free O-H), and 3440 cm^{-1} (medium, broad, hydrogen bonded O-H). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.17-7.70 ppm, 1 proton as a triplet at 4.60 ppm (R_3C-H), 1 proton as a singlet at 3.17 ppm (O-H), 3 protons as a multiplet at 2.24-2.90 ppm (-CH-CH_2), and 6 protons as a broad band at 1.22-2.00 ppm (-CH_2-CH_2-CH_2-). This compound was used as an analytical specimen with no further preparation.

Anal. Calcd. for $C_{18}H_{20}O_2$: C, 80.6; H, 7.5. Found: C, 80.6; H, 7.2.

1-(4-Biphenylyl)-5,6-epoxyhexyl Hydrogen Succinate (IXe). - The ester was prepared from the aforementioned epoxyalcohol XXVII as previously described for the butenyl hydrogen glutarate VIIIb. This provided 560 mg of clear, golden, viscous oil which was composed of a mixture of the desired ester and starting epoxyalcohol XXVII as determined by infrared and nmr spectra. A 157 mg portion of this oil was dissolved in 1 ml of dry chloroform and chromatographed on 10 g of washed silicic acid (196). The starting epoxyalcohol (51 mg) was removed from the column by elution with 20% ether in petroleum ether. Further elution with 50% ether in petroleum ether afforded 78 mg of hydrogen succinate IXe as a clear, colorless oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1720 cm^{-1} (strong, C=O), and 1730 cm-1 (strong, C=O). The nmr spectrum ($CDCl_3$) showed maxima for 1 proton as a broad singlet at 8.38 ppm (-COOH),

9 aromatic protons at 7.23-7.74 ppm, 1 proton as a triplet at 5.83 ppm (R_3C-H), 4 protons as a sharp singlet at 2.64 ppm (succinate methylenes) superimposed on 3 protons as a multiplet at 2.37-2.98 ppm (-CH(O)-CH_2), and 6 protons as a broad band at 1.27-2.12 ppm (-CH_2-CH_2-CH_2-). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-5,6-epoxyhexyl Hydrogen Succinate (IXe-S). - The salt of the aforementioned hydrogen succinate IXe was prepared as previously described from 283 mg (0.77 mmole) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt IXe-S as a white solid, mp 105-107°.

Anal. Calcd. for $C_{30}H_{34}N_2O_5S$: C, 67.39; H, 6.4; N, 5.2. Found: C, 67.5; H, 6.4; N, 5.2.

1-(4-Biphenylyl)-5,6-epoxyhexyl Hydrogen Glutarate (IXf). - The ester was prepared from 891 mg (3.31 mmoles) of the aforementioned epoxyalcohol XXVII as previously described for the butenyl hydrogen glutarate VIIIb to give 340 mg (27%) of hydrogen glutarate IXf as a clear, yellow viscous oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1715 cm^{-1} (strong, C=O), 1730 cm^{-1} (strong, C=O). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.16-7.71 ppm, 1 proton as a broad singlet at 6.66 ppm (-COOH), 1 proton as a singlet at 4.67 ppm (R_3C-H), and 15 protons as broad intermingled multiplets at 0.83-3.00 ppm (epoxyalkyl

and glutarate methylenes). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-5,6-epoxyhexyl Hydrogen Glutarate (IXf-S). - The salt of the aforementioned hydrogen glutarate IXf was prepared as previously described from 165 mg (0.43 mmoles) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether gave an analytical sample of S-benzylthiuronium salt IXf-S as a white solid, mp 79-82°.

Anal. Calcd. for $C_{31}H_{36}N_2O_5S \cdot \frac{1}{2}H_2O$: C, 66.8; H, 6.4; N, 5.0. Found: C, 66.9; H, 6.4; N, 5.3.

1-Bromo-2-butyne (XXVIII). Method A. - The method of Sharifkanov and Skhmedova (177) was used. To a round-bottomed flask fitted with a magnetic stirrer, pressure compensating dropping funnel, and drying tube was added 8.9 g (33 mmoles) of phosphorous tribromide and 0.1 ml of pyridine. To the resulting solution was added in a dropwise manner with stirring and cooling in an ice-sodium chloride bath over a period of 1 hour a solution of 7.0 g (0.1 mole) of 2-butyn-1-ol (Research Organic/Inorganic Chemical Corporation) and 0.1 ml of pyridine. Following the addition, the cloudy brown reaction solution was stirred at room temperature for 1.5 hours, then distilled to give 5.90 g (44%) of 1-bromo-2-butyne (XXVIII) as a clear, colorless liquid, bp 84-87° (150 mm), literature (177) bp 80° (130 mm). The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 2235 cm^{-1} (-C≡C-). The nmr spectrum ($CDCl_3$) showed maxima

for 2 protons as a quartet at 3.94 ppm ($\equiv C-CH_2-Br$) and 3 protons as a triplet at 1.89 ppm ($CH_3-C\equiv$).

1-Bromo-2-butyne (XXVIII). Method B. - Slight modifications in the method of Black et al. (179) and of Coe et al. (180) were used. Thus, to a round-bottomed flask equipped with a magnetic stirrer, pressure compensating dropping funnel, and drying tube were added 16.0 g (0.05 mole) of triphenylphosphite (Aldrich) and 50 ml of dry ether. To the resulting solution was added in a dropwise manner over a period of 0.5 hour with stirring and cooling in an ice bath 8.00 g (0.05 mole) of liquid bromine (Matheson, Coleman and Bell) and the resulting mixture was stirred at room temperature for 20 minutes. To the above solution of triphenylphosphite dibromide was added, in a dropwise manner with stirring and cooling in an ice bath, over a period of 1 hour a solution of 3.50 g (0.05 mole) of 2-butyn-1-ol (Research Organic/Inorganic Chemicals Corporation) and 3.95 g (0.05 mole) of pyridine. On completion of the addition, the reaction mixture was stirred at room temperature for 21 hours, filtered, and the white solid was washed with ether. The filtrate was poured into an ether-water mixture, the phases separated, and the aqueous phase extracted twice with ether. The combined organic phases were washed with 5% sodium hydroxide solution, water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure and distillation of the residue

afforded 3.83 g (58%) of 1-bromo-2-butyne (XXVIII) as a clear, colorless liquid, bp 84-88° (153 mm), literature (177) bp 80° (130 mm). The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 2235 cm^{-1} (strong, C≡C). The nmr spectrum ($CDCl_3$) showed maxima for 2 protons as a quartet at 3.94 ppm ($Br-CH_2-C\equiv C-$) and 3 protons as a triplet at 1.88 ppm ($-C\equiv C-CH_3$).

Attempted preparation of 1-(4-Biphenylyl)-3-pentyn-1-ol (XXIX). Method A. - To a three-necked flask fitted with a mechanical stirrer, reflux condenser, gas inlet tube, pressure compensating dropping funnel, and drying tube was added 215 mg (0.009 g-atom) of magnesium turnings and the system was flame-dried under a constant stream of dry nitrogen. When the system had cooled, a small crystal of iodine and 0.5 ml of dry ether were added to the flask. To the resulting mixture was added over a period of 10 minutes, with stirring, a solution of 961 mg (7.2 mmoles) of previously prepared 1-bromo-2-butyne (XXVIII) dissolved in 1 ml of ether. To the Grignard reagent was added in a dropwise manner, at a rate sufficient to maintain gentle reflux, a solution of 1.27 g (7.0 mmoles) of 4-biphenylcarboxaldehyde, mp 57-59° (Aldrich), dissolved in 5 ml of dry ether and 5 ml of dry tetrahydrofuran; then the reaction mixture was stirred at room temperature for 1.5 hours. To the mixture was added in a slow dropwise manner with stirring at room temperature a solution of saturated ammonium chloride which had been adjusted to pH 7 with

concentrated ammonium hydroxide solution. When no more precipitate formed, the mixture was filtered, the solid washed with ether and the cloudy, yellow filtrate was washed with 5% hydrochloric acid solution, water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 1.24 g of a viscous red-brown oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 2825 cm^{-1} (medium, aldehyde C-H), 2730 cm^{-1} (medium, aldehyde C-H), and 1695 cm^{-1} (strong, C=O) and was identical to the infrared spectrum of an authentic sample of 4-biphenylcarboxaldehyde (Aldrich).

Attempted preparation of 1-(4-Biphenylyl)-3-pentyn-1-ol (XXIX). Method B. - The method of Henbest et al. (178) was used. To a flask equipped with a magnetic stirrer were added 1.30 g (0.02 g-atom) of zinc powder (Fisher) and 10 ml of a 5% hydrochloric acid solution. The zinc powder was etched by stirring the resulting mixture for 15 minutes at room temperature, then washed 3 times with water, twice with absolute methanol, and 3 times with ether by allowing the zinc powder to settle after a brief period of stirring and decanting the resulting supernatant solution before the next wash. Finally, the zinc was covered with dry benzene which was evaporated under a gentle stream of dry nitrogen until a total volume of 0.5-1.0 ml was obtained. The flask was fitted with a reflux condenser, pressure compensating dropping funnel, and to the system were added over a period of 15 minutes, with stirring initially at

room temperature, a solution of 798 mg (6.0 mmoles) of the previously described 1-bromo-2-butyne (XXVIII), 1.09 g of 4-biphenylcarboxaldehyde (6.0 mmoles), mp 57-59° (Aldrich), and 3.8 ml of dry tetrahydrofuran. After approximately 1 ml of the above solution had been added to the zinc slurry a trace of mercuric chloride was added and the remainder of the addition was completed at a rate which allowed maintenance of steady reflux. After completion of the addition, the reaction mixture was refluxed for 0.5 hour then poured onto 50 ml of ice-10% acetic acid slurry. The resulting mixture was extracted with ether and the organic phase was washed with water, 5% sodium hydroxide solution, water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 905 mg of a light yellow liquid which was not completely characterized since it proved to be an undesired product as shown below by the spectral data. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3590 cm^{-1} (medium, free O-H), 3410 cm^{-1} (broad, medium, hydrogen bonded O-H), and 1963 cm^{-1} (medium, allene). The conspicuous absence of infrared absorption in the 2100-2300 cm^{-1} region and the presence of the sharp 1963 cm^{-1} absorption indicated that an allene was obtained rather than the desired alkynyl alcohol.

1-Phenyl-3-butyn-1-ol (XXX). A Model System. - The method of Läuger et al. (181) was used. To a three-necked flask equipped with a magnetic stirrer, pressure compensating

dropping funnel, and drying tube were added 1.78 g (0.066 g-atom) of aluminum, 25 ml of dry tetrahydrofuran, and a trace of mercuric chloride. The aluminum was prepared by cutting 0.13 mm thick aluminum sheet into 0.5 cm X 4.0 cm strips which were rolled into small cylinders. To the above mixture was added in a dropwise manner with stirring over a period of 0.5 hour a solution of 11.9 g (0.10 mole) of 1-bromo-2-propyne (Aldrich) dissolved in 25 ml of tetrahydrofuran. During the addition the reaction mixture was maintained at 25-30° and on completion of the addition it was stirred at 35-40° for 0.5 hour. To the resulting black mixture was added, in a dropwise manner over a period of 40 minutes while stirring at 30°, a solution of 10.6 g (0.10 mole) of benzaldehyde (Matheson, Coleman and Bell) dissolved in 50 ml of dry ether. The resulting opaque, brown mixture was stirred at 35-40° for 0.5 hour, then poured onto 200 ml of an ice-water slurry and stirred until the mixture reached room temperature. The phases were separated and the aqueous phase was washed with ether. The organic phase was washed with water until the washings were pH 6, then with saturated sodium chloride solution and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure provided a brown liquid which was distilled to afford 8.08 g (55%) of alcohol XXX as a clear, colorless liquid, bp 77-80° (0.8 mm), literature (181) bp 85-87° (0.5 mm). The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3590 cm^{-1} (strong, free O-H), 3420 cm^{-1} (strong, broad,

hydrogen bonded O-H), 3300 cm^{-1} (strong, alkyne C-H), and 2110 cm^{-1} (weak, C≡C). The nmr spectrum ($CDCl_3$) showed maxima for 5 aromatic protons at 7.47 ppm, 1 proton as a triplet at 4.75 ppm (R_3C-H), 1 proton as a singlet at 3.52 ppm (R-O-H), 2 protons as a pair of doublets centered at 2.52 ppm ($-CH_2-$), and 1 proton as a triplet at 1.97 ppm (-C≡C-H).

Attempted preparation of 1-(4-Biphenylyl)-3-pentyn-1-ol (XXIX). Method C. - The method of Läuger (181) as previously described for the model system 1-phenyl-3-butyn-1-ol (XXX) was followed using 189 mg (0.007 g-atom) of aluminum in 10 ml of tetrahydrofuran and 1.33 g (0.01 mole) of the previously described 1-bromo-2-butyne (XXVIII) in 10 ml of tetrahydrofuran to generate the alkynyl aluminum complex. Failure of the complex to form was indicated by the almost complete lack of degradation of the aluminum surface after four hours at 45°. After a total of 20 hours at 45° evidence of aluminum degradation was observed and to the resulting mixture was added over a period of 15 minutes, while stirring at room temperature, a solution of 1.82 g (0.01 mole) of 4-biphenylcarboxaldehyde mp 52-55° (Aldrich), dissolved in 10 ml of tetrahydrofuran. The resulting mixture was stirred for 3 hours at room temperature then poured onto an ice-water slurry and stirred until the mixture reached room temperature. The phases were separated and the aqueous phase was washed with ether. The combined organic phases were washed with

water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 1.84 g of an opaque, black liquid. The infrared spectrum of this material showed λ_{max}^{neat} 2825 cm^{-1} (medium, aldehyde C-H), 2730 cm^{-1} (medium, aldehyde C-H), and 1700 cm^{-1} (strong, C=0) indicating that the desired alkynyl alcohol had not been obtained.

Trimethylsulfoxonium Iodide (XXXI). - The method of Kuhn and Trischmann (182) was used. To a one-necked, round-bottomed flask equipped with gas inlet tube, reflux condenser, heating mantle and magnetic stirrer were added 96 g (1.23 moles) of dimethylsulfoxide (Fisher) and 410 g (2.9 moles) of methyl iodide (Columbia Organic Chemicals Co.) and the resulting solution was maintained at reflux for 72 hours under a nitrogen atmosphere. The reaction mixture was then cooled to room temperature, filtered, and the solid was washed with chloroform. The resulting white powder was recrystallized twice from hot water to give large, white needles which were crushed and dried in a vacuum desiccator to provide 52.9 g (19.5%) of trimethylsulfoxonium iodide (XXXI) as a light brown solid, mp 195° (dec), literature (182) mp 200° (dec). The infrared spectrum was identical to that published by Kuhn and Trischman (182).

1-Phenyl-1,2-epoxyethane (XXXII). A Model System. - The method developed by Corey and Chaykovsky (183) was used. To a three-necked flask fitted with reflux condenser,

pressure compensating dropping funnel, magnetic stirrer and gas inlet system (184) was added 1.26 g (0.03 mole) of sodium hydride as a 57% oil dispersion. The sodium hydride was washed 3 times with petroleum ether (30-60°) by stirring and allowing the powder to settle while the system was flushed with a continuous stream of dry nitrogen. After each wash the petroleum ether was decanted and finally the sodium hydride was dried by alternately evacuating and flushing the system with dry nitrogen. To the remaining powder there was added 30 ml of tetrahydrofuran; then, while the system was maintained under positive nitrogen pressure, 6.66 g (0.03 mole) of the previously described trimethylsulfoxonium iodide (XXXI) was added as a solid over a period of 15 minutes while stirring at room temperature. The resulting mixture was heated at reflux for 2 hours; then, to the opaque white suspension was added, while stirring at reflux, over a period of 1.5 hours a solution of 2.86 g (0.027 mole) of benzaldehyde (Matheson, Coleman and Bell) in 30 ml of tetrahydrofuran. The resulting mixture was stirred at reflux for an additional 1 hour followed by removal of the solvent under reduced pressure until approximately 15 ml remained. The residue was poured into 75 ml of water and extracted with ether. The combined ether extracts were washed with saturated sodium chloride solution and dried over anhydrous sodium sulfate. Removal of the solvent at reduced pressure provided an orange liquid which was distilled to give

0.95 g (29%) of 1-phenyl-1,2-epoxyethane (XXXII) as a clear, colorless liquid, bp 26-28° (1 mm). The infrared spectrum was identical to that of an authentic sample of the desired epoxide (Aldrich). The nmr spectrum ($CDCl_3$) showed maxima for 5 aromatic protons at 7.21 ppm, 1 proton as a pair of doublets centered at 3.77 ppm (H_c Figure 13, R = H), 1 proton as a pair of doublets centered at 3.08 ppm (H_b Figure 13, R = H), and 1 proton as a pair of doublets centered at 2.74 ppm (H_a Figure 13, R = H). The nmr spectrum was identical to the spectrum of an authentic sample of styrene oxide (Aldrich).

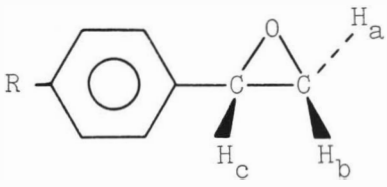


Figure 13: Proton positions in styrene oxide.

1-(4-Biphenylyl)-1,2-epoxyethane (XXXIII). Method A. - The method of Corey and Chaykovsky (183) as previously described for the model system 1-phenyl-1,2-epoxyethane (XXXII) was employed. The ylide was generated from 1.29 g of sodium hydride as a 56% dispersion in oil (0.03 mole) and 6.60 g (0.03 mole) of trimethylsulfoxonium iodide (Aldrich). To the resulting ylide suspension was added, in a dropwise manner, with stirring over a period of 45 minutes at reflux temperature a solution of 5.46 g (0.03

mole) of 4-biphenylcarboxaldehyde, mp 52-55° (Aldrich), dissolved in 35 ml of tetrahydrofuran. Following the addition, the reaction mixture was stirred and maintained at reflux for 1.25 hours; then the solvent was removed under reduced pressure until approximately 25 ml of the mixture remained. The residue was poured into 100 ml of water, extracted 3 times with ether and the combined organic phases were washed with water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 5.25 g of an oily, yellow solid, mp 39-45°, which resisted recrystallization attempts. A 1.02 g portion of this material was dissolved in 1.5 ml of chloroform, and chromatographed on 25 g of 60-100 mesh Florisil (Fisher). Elution with 600 ml of petroleum ether afforded 369 mg (representing an overall reaction yield of 32%) of the desired epoxyethane XXXIII as a white solid, mp 86-89°. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1245 cm^{-1} (medium, epoxide) and 1072 cm^{-1} (medium, epoxide). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.20-7.70 ppm, 1 proton as a pair of doublets centered at 3.85 ppm (H_c Figure 13, R = phenyl), 1 proton as a pair of doublets centered at 3.14 ppm (H_b Figure 13, R = phenyl), and 1 proton as a pair of doublets centered at 2.80 ppm (H_a Figure 13, R = phenyl). This material was employed as an analytical sample with no further preparation.

Anal. Calcd. for $C_{14}H_{12}O$: C, 85.7; H, 6.2. Found:

C, 85.7; H, 6.4.

Preparation of Diethylaluminum Chloride Stock Solution. - The method of Nagata and Yoshioka (185) was employed. A cylinder of diethylaluminum chloride (Alpha-Ventron) was connected with tygon tubing to a two-way stopcock which was connected with tygon tubing to a straight drying tube. The drying tube was fitted to a straight vacuum take-off adapter which was fitted to a round-bottomed flask which had been equipped with a magnetic stirrer (Figure 14). To the flask was added 150 ml of petroleum ether (60-70^{o}) and the system was alternately evacuated and flushed with dry nitrogen. The reagent cylinder valve was opened and 38.3 g (0.32 mole) of diethylaluminum chloride was slowly drained into the stirred petroleum ether at room temperature. The cylinder valve was closed and the system was alternately evacuated and flushed with dry nitrogen, then the flask was disconnected from the system and a rubber septum was quickly inserted and wired onto the neck of the flask. The resulting clear, colorless solution contained approximately 234 mg (1.94 mmoles) of diethylaluminum chloride per ml of solution.

Attempted Synthesis of 1-Phenyl-3-pentyn-1-ol (XXXV). A Model System. - The methods of Fried et al. (186) and of Hooz and Layton (187) were modified. To a round-bottomed flask equipped with a magnetic stirrer, straight vacuum take-off, and pressure compensating dropping funnel equipped with a rubber septum were added 1.84 g (40 mmoles) of

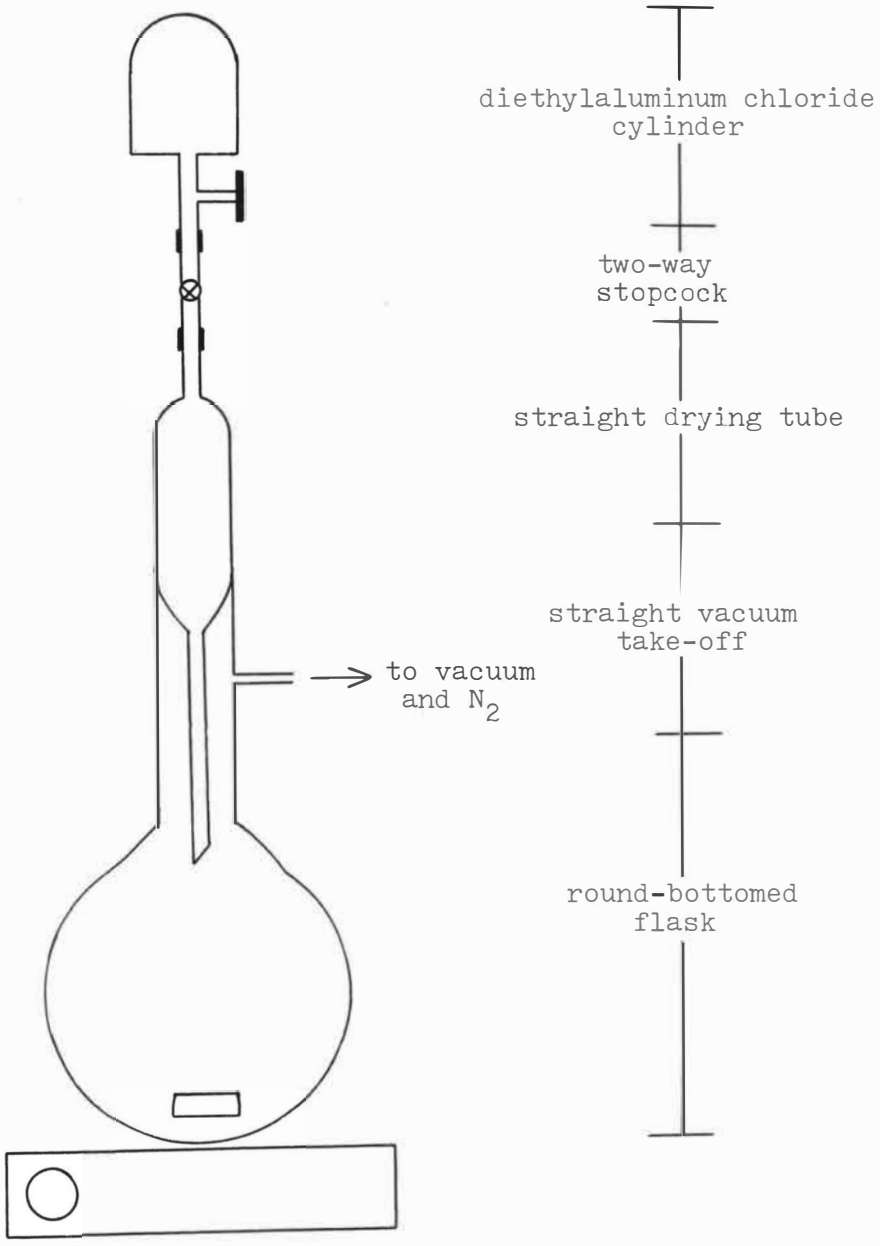


Figure 14: Aparatus for preparation of diethylaluminum chloride stock solution.

propynyllithium (Alpha-Ventron) and 25 ml of dry petroleum ether (60-70°). The system was alternately evacuated and flushed with dry nitrogen and then 21 ml (approximately 41 mmoles) of the previously described stock solution of diethylaluminum chloride (XXXIV) was transfered to the dropping funnel by means of a nitrogen filled syringe. The alane was added to the propynyllithium suspension in a dropwise manner over a period of 20 minutes with stirring and cooling in an ice bath; then the reaction mixture was stirred at room temperature for 1 hour. To the resulting solution was added, in one portion, 10 ml of ether followed by the addition, over a period of 15 minutes with stirring and cooling in an ice bath, of a solution of 2.40 g (20 mmoles) of styrene oxide (Aldrich) dissolved in 10 ml of dry petroleum ether (60-70°). The resulting mixture was stirred at room temperature for 19 hours, then added to an Erlenmeyer flask and, with stirring and cooling in an ice bath, a saturated solution of ammonium chloride which had been adjusted to pH 7 with concentrated ammonium hydroxide solution was added in a dropwise manner until no further precipitation was observed. The resulting mixture was poured into a mixture of ether and water, the phases separated and the aqueous phase was extracted with ether. The organic phase was washed with water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure gave 2.64 g (81%) of clear, golden yellow mobile oil. The infrared

spectrum showed $\lambda_{max}^{CHCl_3}$ 3585 cm^{-1} (medium, free O-H), 3450 cm^{-1} (medium, broad, hydrogen bonded O-H) and 2235 cm^{-1} (weak, -C≡C-). The nmr spectrum ($CDCl_3$) showed maxima for 5 aromatic protons at 7.26 ppm, 2 protons as a sharp singlet at 3.67 ppm ($-CH_2-O-$) superimposed on 1 proton as a multiplet at 3.37-3.96 ppm (R_3C-H), 1 proton as a singlet at 2.17 ppm (O-H), and 3 protons as a doublet at 1.83 ppm ($-C\equiv C-CH_3$). These spectral data are not consistent with the structure of the desired alkynyl alcohol XXXV and suggest that the reaction product was probably 2-phenyl-3-pentyn-1-ol (XXXVI).

2-Phenyl-1-pentanol (XXXVII). - To a Parr hydrogenation bottle was added 960 mg (6.0 mmoles) of the previously described compound suspected to be 1-phenyl-3-pentyn-1-ol (XXXV), 70 mg of 5% palladium on charcoal (Englehard Industries), and 30 ml of absolute ethanol and the mixture was shaken on a Parr hydrogenator under 2.11 kg/cm^2 hydrogen pressure for 18 hours at room temperature. The mixture was filtered through a Celite pad and the pad was washed with ten ml of absolute ethanol. Removal of the solvent under reduced pressure afforded 852 mg of dark brown, mobile oil. Molecular distillation of 242 mg of this material provided 154 mg (an overall yield of 55%) of alcohol XXXVII as a clear, colorless mobile oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3600 cm^{-1} (medium, free O-H), and 3460 cm^{-1} (medium, broad, hydrogen bonded O-H). The nmr spectrum ($CDCl_3$) showed maxima for 5 aromatic

protons at 7.20 ppm, 2 protons as a doublet at 3.68 ppm ($-CH_2-O-$), 1 proton as a multiplet centered at 2.74 ppm (R_3C-H), 1 proton as a singlet at 1.82 ppm (-O-H), and 7 protons as multiplets at 0.63-1.78 ppm ($-CH_2-CH_2-CH_3$).

Anal. Calcd. for $C_{11}H_{16}O$: C, 80.4; H, 9.8. Found: C, 80.0; H, 9.7.

1-Phenyl-3-pentyn-1-ol (XXXV). A Model System. Method B. - Minor modifications were made in the method of Fries (188). To a round-bottomed flask equipped with magnetic stirrer, pressure compensating dropping funnel and nitrogen atmosphere were added 966 mg (21 mmoles) of propynyllithium (Alpha-Ventron) and 15 ml of dry dimethylsulfoxide. To the resulting suspension was added in a dropwise manner with stirring at room temperature over a period of 20 minutes a solution of 1.28 g (10.7 mmoles) of the previously described 1-phenyl-1,2-epoxyethane (XXXII), dissolved in 5 ml of dry dimethylsulfoxide. The opaque, black reaction mixture was stirred for 15 hours at room temperature, then slowly poured into 150 ml of stirred ice-water slurry and the resulting mixture was extracted with ether. The organic phase was washed with water, saturated sodium chloride solution and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 659 mg (38%) of pentynyl alcohol XXXV as a clear, yellow liquid. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3600 cm^{-1} (medium, free O-H), 3450 cm^{-1} (medium, broad, hydrogen bonded O-H), and 2220 cm^{-1} (weak, alkyne).

The nmr spectrum ($CDCl_3$) showed maxima for 5 aromatic protons at 7.27 ppm, 1 proton as a triplet at 4.75 ppm (R_3C-H), 1 proton as a moderately broad band at 2.67 ppm (O-H) which was partially obscured by 2 protons as a multiplet at 2.42-2.65 ppm ($-CH_2-$), and 3 protons as a sharp triplet at 1.75 ppm ($-CH_3$). Several attempts to prepare an analytical specimen of this compound by molecular distillation with a Hickman still were unsuccessful.

1-(4-Biphenylyl)-3-pentyn-1-ol (XXIX). Method D. - The method of Fries (188) was employed. To a round-bottomed flask fitted with a magnetic stirrer, pressure compensating dropping funnel, and nitrogen atmosphere were added 184 mg (4.0 mmoles) of propynyllithium (Alpha-Ventron) and 10 ml of dry dimethylsulfoxide. To the resulting mixture was added, in a dropwise manner, with stirring at room temperature over a period of 10 minutes a solution of 392 mg (2.0 mmoles) of the previously described 1-(4-Biphenylyl)-1,2-epoxyethane (XXXIII) dissolved in 5 ml of dry dimethylsulfoxide. The reaction mixture was stirred at 36-42° for 48 hours, then slowly poured onto 100 ml of an ice-water slurry and extracted with ether. The organic phase was washed with water, a saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure provided 219 mg of clear, dark brown oil which was not structurally clearly defined by spectral means. A portion of this material (164 mg) was dissolved in 0.75 ml of chloroform and chromatographed on

18 g of 60-100 mesh Florisil (Fisher) and eluted in 100 ml fractions. The fractions eluted with 10% ether in petroleum ether afforded 57 mg (16%) of a white solid, mp 109-111°. Recrystallization of this material from petroleum ether (60-70°) provided 43 mg of pentynyl alcohol XXIX as a white solid, mp 110.5-111.5°. The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.15-7.65 ppm, 1 proton as a triplet at 4.82 ppm (R_3C-H), 3 protons as a multiplet at 2.40-2.72 ppm (O-H and $-CH_2-C\equiv$), and 3 protons as a sharp triplet at 1.77 ppm ($-C\equiv C-CH_3$). This material was used as an analytical sample with no further preparation.

Anal. Calcd. for $C_{17}H_{16}O$: C, 86.4; H, 6.8. Found: C, 86.2; H, 6.8.

Triphenylmethylphosphonium Bromide (XXXVIII). - The method of Wittig and Schoellkopf (189) was followed. Approximately 8 ml (0.145 mole) of methyl bromide (J. T. Baker) was distilled into a three-necked round-bottomed flask fitted with a dry ice condenser, gas inlet tube, drying tube, and iso-propyl alcohol-dry ice cooling bath. The clear, colorless liquid was added in one portion to a pressure bottle which contained a solution of 26.2 g (0.10 mole) of triphenylphosphine (Aldrich) dissolved in 28 ml of benzene and was cooled in an ice-salt bath. The stopper was wired into place and the reaction mixture was allowed to reach room temperature and then allowed to remain at this temperature for 48 hours. The resulting

white cake was crushed, slurried with 400 ml of hot benzene, filtered, and air dried for 0.5 hour. Drying in a vacuum oven (water aspirator) at 100° for 20 hours provided 35.0 g (98%) of triphenylmethylphosphonium bromide (XXXVIII) as a white solid, mp 230-231°, literature (189) mp 232-233°.

(4-Biphenylyl)ethene (XXXIX). - The method of Greenwald et al. (190) was used. To a three-necked round-bottomed flask fitted with a gas inlet tube and magnetic stirrer was added 471 mg (11 mmoles) of sodium hydride (Alpha-Ventron) as a 56% dispersion in oil which was washed three times under a nitrogen atmosphere by stirring with petroleum ether (60-70°). After each wash, the powder was allowed to settle and the supernatant solution was decanted. After the final wash the flask was fitted with a reflux condenser and pressure compensating dropping funnel and the system was alternately evacuated and flushed with dry nitrogen until the sodium hydride was dry. In one portion, 25 ml of dimethylsulfoxide was added to the residue and the resulting suspension was heated to 75° while stirring for 50 minutes to give a clear, brown solution of methyl sulfinyl carbanion. The system was cooled in an ice bath and a solution of 3.57 g (10 mmoles) of the previously described triphenylmethylphosphonium bromide (XXXVIII) in 25 ml of dimethylsulfoxide was added in a dropwise manner with stirring over a period of 5 minutes. The resulting ylide was stirred at room temperature for 15 minutes followed by the dropwise addition over a period of 5 minutes of

1.82 g (10 mmoles) of 4-biphenylcarboxaldehyde, mp 57-59° (Aldrich), dissolved in 10 ml of dimethylsulfoxide. The dark green reaction solution was stirred at room temperature for 2 hours, at 65° for 1 hour, then cooled to room temperature and poured onto 150 ml of an ice-water slurry. The resulting mixture was extracted with 200 ml of petroleum ether (60-70°) and the emulsion which formed was extracted in 25 ml portions with 200 ml of ether. The organic phase was washed with water, saturated sodium chloride solution and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure provided 2.02 g of light yellow solid which was dissolved in 3.0 ml of chloroform and chromatographed on 50 g of neutral alumina (80-200 mesh, Brockman Activity I). Elution with two liters of petroleum ether afforded 725 mg (40%) of alkene XXXIX as a white solid, mp 118.5-119.5°, literature (191) mp 120-122°. The nmr spectrum showed maxima for 9 protons of aromatic nature at 7.18-7.65 ppm, 1 proton as a pair of doublets centered on 6.74 ppm (Ar-CH=), 1 proton as a doublet at 5.70 ppm (trans =C-H, J = 18 Hz), and 1 proton as a doublet at 5.21 ppm (cis =C-H, J = 10 Hz). This material was used as an analytical specimen with no further preparation.

Anal. Calcd. for $C_{14}H_{12}$: C, 93.3; H, 6.7. Found: C, 93.5; H, 6.8.

1-(4-Biphenylyl)-1,2-epoxyethane (XXXIII). Method B. - The method described by Fieser and Fieser (176) was used.

To a round-bottomed flask equipped with a pressure compensating dropping funnel and magnetic stirrer and which contained 520 mg (2.87 mmoles) of the previously described (4-biphenylyl)ethene (XXXIX), mp 118.5-119.5°, in 20 ml of dry chloroform was added, in a dropwise manner over a period of 1 hour with stirring and cooling in an ice bath, a solution of 875 mg (4.3 mmoles) of 85% m-chloroperbenzoic acid dissolved in 45 ml of chloroform. The resulting clear, colorless solution was stirred at room temperature for 20 hours, then with 50 ml of a 10% sodium sulfite solution until a negative starch-iodide test was observed. The phases were separated and the organic phase was washed with 5% sodium hydrogen carbonate solution, water, saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure provided 670 mg of a waxy white solid, mp 59-74°. A 559 mg portion of this material was chromatographed on 27 g of 60-100 mesh Florisil (Fisher). Elution with 1% ether-petroleum ether afforded 269 mg (an overall yield of 57%) of epoxide XXXIII as a white solid, mp 90-91°. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 1247 cm^{-1} (medium, epoxide), 877 cm^{-1} (sharp, strong, epoxide), and 837 cm^{-1} (sharp, strong, epoxide). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.18-7.71 ppm, 1 proton as a pair of doublets centered at 3.86 ppm (Ar-$\overset{|}{C}$-H), 1 proton as a pair of doublets centered at 3.12 ppm (H_b Figure 13, R = phenyl), and 1 proton as a pair of doublets centered at 2.78 ppm

(H_a Figure 13, R = phenyl). This material was used as an analytical specimen with no further preparation after chromatography.

Anal. Calcd. for $C_{14}H_{12}O$: C, 85.7; H, 6.2. Found: C, 85.7; H, 6.4.

1-(4-Biphenylyl)-2-butyn-1-ol (XL). - A modification of the procedure of Smith and Kuehn (192) was used. To a three-necked flask equipped with a magnetic stirrer, pressure compensating dropping funnel, nitrogen atmosphere and oil bath were added 506 mg (11 mmoles) of propynyllithium (Alpha-Ventron) and 13 ml of tetrahydrofuran which had been freshly distilled from lithium aluminum hydride. To the resulting suspension was added, in a dropwise manner over a period of 10 minutes with stirring at 40°, a solution of 1.82 g (10 mmoles) of 4-biphenylcarboxaldehyde (Aldrich, base washed) dissolved in 2.5 ml of tetrahydrofuran which had been freshly distilled from lithium aluminum hydride. Following the addition, the reaction mixture was stirred at 40° for 5 minutes, then placed into an ice bath and 25 ml of ether was added in one portion followed by 15 ml of saturated ammonium chloride solution which had been adjusted to pH 7 with concentrated ammonium hydroxide solution. The resulting mixture was stirred for 5 minutes, the phases were separated and the organic phase was washed once with water, with saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure provided 1.79 g of a yellow-brown

solid which was dissolved in 4.0 ml of chloroform and chromatographed on 80 g of 60-100 mesh Florisil (Fisher). Elution with 10% ether-petroleum ether afforded 1.04 g (47%) of butynyl alcohol XL as a white solid. Recrystallization from petroleum ether (60-70°) afforded a white solid, mp 88-90°. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 3590 cm^{-1} (medium, free O-H), 3420 cm^{-1} (medium, broad, hydrogen bonded O-H), 2280 cm^{-1} (weak, C≡C), and 2220 cm^{-1} (weak, C≡C). The nmr spectrum ($CDCl_3$) showed maxima for 9 aromatic protons at 7.24-7.63 ppm, 1 proton as a quartet at 5.41 ppm (R_3C-H), 1 proton as a broad singlet at 2.42 ppm (O-H), and 3 protons as a doublet at 1.83 ppm ($-CH_3$). Two recrystallizations of this compound from petroleum ether (60-70°) afforded an analytical specimen of butynyl alcohol XL as a white solid, mp 88-90°.

Anal. Calcd. for $C_{16}H_{14}O$: C, 86.5; H, 6.4. Found: C, 86.2; H, 6.2.

1-(4-Biphenylyl)-2-butynyl Hydrogen Succinate (XLI). - The ester was prepared from the aforementioned butynyl alcohol XL as previously described for the butenyl hydrogen glutarate VIIIb to give 1.12 g of clear, brown, very viscous oil. A 515 mg portion of this material was chromatographed on 25 g of washed silicic acid (196). Elution with 50% ether in petroleum ether afforded 263 mg (33%) of hydrogen succinate XLI as a beautiful, clear, colorless oil. The infrared spectrum showed $\lambda_{max}^{CHCl_3}$ 2290 cm^{-1} (weak, C≡C), 2230 cm^{-1} (weak, C≡C), 1735 cm^{-1} (strong, C=O), and 1713 cm^{-1}

(strong, C=O). The nmr spectrum showed maxima for 1 proton as a broad singlet at 9.65 ppm (-COOH), 9 aromatic protons at 7.21-7.72 ppm, 1 proton as a quartet at 6.49 ppm (R_3C-H), 4 protons as a sharp singlet at 2.64 ppm ($-CH_2-CH_2-$), and 3 protons as a doublet at 1.90 ppm ($-CH_3$). This compound was analytically characterized as described below.

S-Benzylthiuronium Salt of 1-(4-Biphenylyl)-2-butynyl Hydrogen Succinate (XLI-S). - The salt of the aforementioned hydrogen succinate XLI was prepared as previously described from 269 mg (0.84 mmole) of the ester. Two recrystallizations of the crude salt from ethyl acetate-petroleum ether (60-70°) afforded an analytical sample of S-benzylthiuronium salt XLI-S as a white solid, mp 112-114°.

Anal. Calcd. for $C_{28}H_{28}N_2O_4S$: C, 68.9; H, 5.8; N, 5.7. Found: C, 68.7; H, 5.7; N, 5.7.

C. Preparation of Rat Liver Microsomes

The method of Shapiro and Rodwell (82) was used. Male Sprague-Dawley rats (approximately 100 g) obtained from Holtzman were fed Purina Laboratory Chow (Ralston Purina Company) ad libitum and housed for at least 4 days with light from 7 AM to 7 PM and dark from 7 PM to 7 AM. A group of eight rats was used for each microsome preparation. Between 12:05 and 12:25 AM the animals were stunned by a sharp blow to the head, decapitated, and the livers were excised into ice-cold ESM solution at pH 6.0 (ESM solution is composed of 30mM ethylenediamine tetraacetic acid, 70 mM sodium

chloride and 10 mM β-mercaptoethanol). The mass of the livers was determined; they were minced and forced through a tissue press into 2.5 ml of ice-cold ESM solution per gram of liver. The resulting tissue mixture was homogenized in a Potter-Elvehjem homogenizer with five strokes of a Teflon pestle. The homogenate was centrifuged twice for 15 minutes at 12,000 X g and the pellet was discarded each time. The supernatant solution was centrifuged for 1 hour at 48,000 X g, the resulting supernatant was discarded, and the microsomal pellets were frozen in a dry ice-iso-propyl alcohol bath. The tubes were sealed with Parafilm and stored at -60° for up to three weeks.

D. Assay of Enzyme Inhibitors

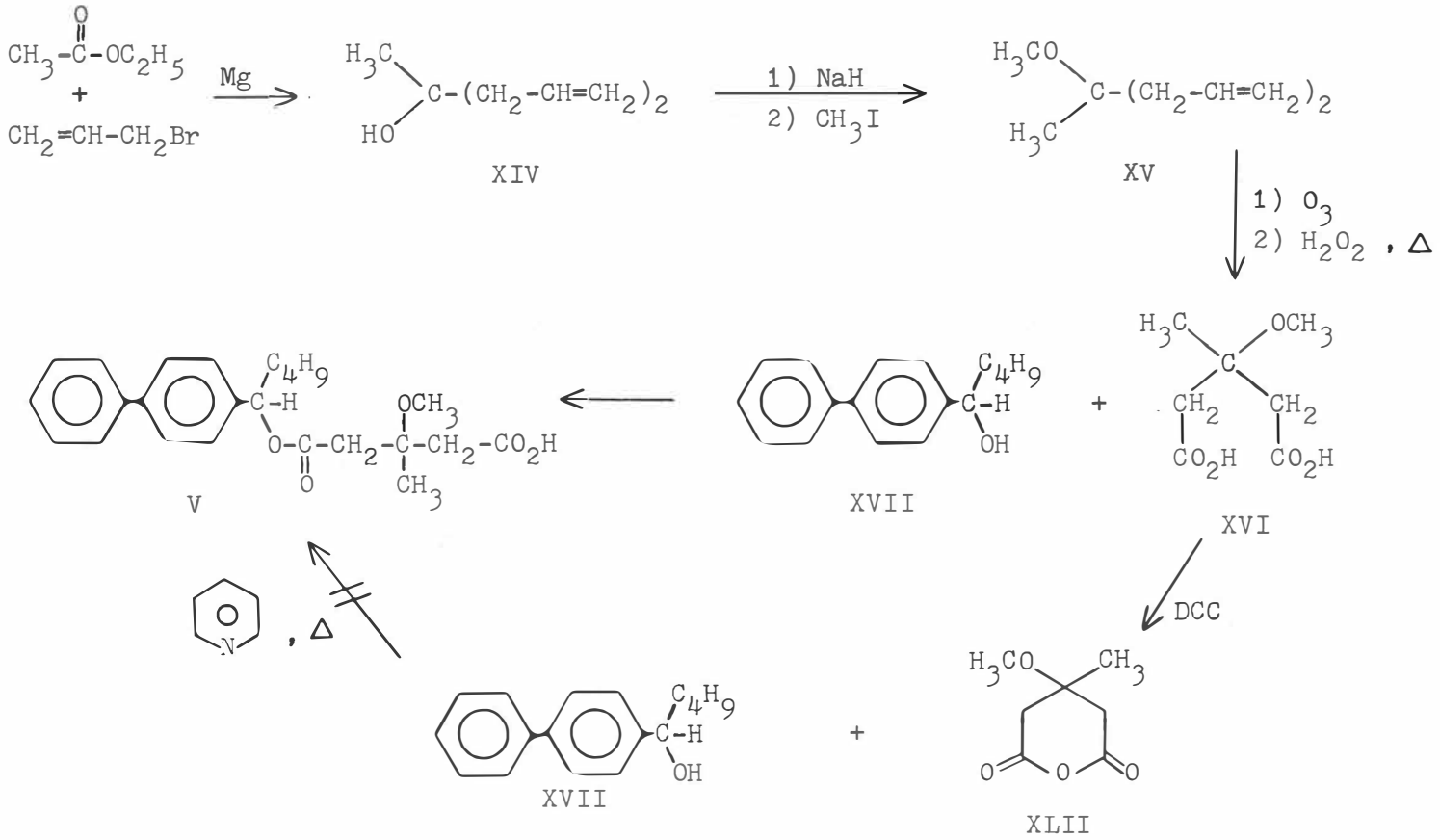
A slight modification of the method developed by Rodwell (82, 193) was employed. A portion of the rat liver microsome preparation was suspended in 1.5 ml of the previously described ESM solution, pH 6.8. Each assay mixture contained 9.7 μ moles of $NADP^{+}$ (Sigma), 59.7 μ moles of glucose-6-phosphate dehydrogenase Type XII from torula yeast (Sigma), 0.15 ml of microsome suspension, 0.1 ml of ethylene glycol monoethyl ether (Fisher) and the appropriate concentration of inhibitor in a total volume of 2.9 ml. The total assay mixture contained 1.6-2.1 mg of protein as determined by the method of Slakey et al. (84). The enzyme reaction was started by the addition of 0.1 ml of solution containing 94 nmoles of 3-C^{14}-HMG-CoA. The assay system was incubated

for 30 minutes with shaking at 37°, then the reaction was stopped by the addition of 0.2 ml of concentrated hydrochloric acid and a known amount of H^3-mevalonic acid as the mevalonolactone (New England Nuclear) was added as an internal standard. The resulting mixture was set aside for 16 hours at room temperature; then it was saturated with anhydrous sodium sulfate and the mevalonolactone was extracted with ether. The organic phase was evaporated to dryness under a stream of nitrogen; the residue was dissolved in 0.2 ml of acetone and applied as a streak to an activated (30 minutes at 100°) Mylar backed silica gel G thin layer chromatography sheet (Eastman). The chromatograms were developed with benzene-acetone, 1/1 (v/v). The area corresponding to mevalonolactone (R_f = 0.6-1.0) was removed with a razor blade and placed into a liquid scintillation vial for counting. To account for variations of enzyme activity in microsomes from different preparations, one inhibitor, 1-(4-biphenylyl)-n-pentyl hydrogen succinate (III, m = 2), was assayed concurrently with each of the other inhibitors. Therefore, all inhibition activities were directly compared with a compound of known inhibitory properties.

E. Discussion of Experimental Procedures

The preparation of 1-(4-biphenylyl)-n-pentyl hydrogen 3-Methyl-3-methoxyglutarate (V) is described below. The approaches used in the preparation of this compound are illustrated in scheme I. Synthesis of the 4,4-disubstituted heptadiene XIV was smoothly accomplished in 77-89% yields. The use of the double addition method developed by Tschesche and Machleidt (165) provided a means of minimizing side reactions of the highly reactive allyl Grignard reagent. The infrared spectrum of this compound exhibited absorption bands at 3400 cm^{-1} due to the O-H stretching vibration of the alcohol and at 1647 cm^{-1} due to double bond vibration. Further evidence that the desired unsaturated alcohol was obtained was shown by the nuclear magnetic resonance (nmr) spectrum. Multiplets at 5.60-6.37 ppm and at 4.83-5.16 ppm proved the presence of the terminal vinyl group, and observation of a doublet at 2.17 ppm showed the presence and position of the methylene groups. Supporting the infrared evidence, the hydroxyl group was observed as a sharp singlet at 2.38 ppm which was destroyed by exchange with deuterium oxide.

The resulting tertiary alcohol XIV was alkylated with methyl iodide to give the corresponding ether XV in a yield of 65%. Absence of hydroxyl absorption and survival of the double bond absorption in the infrared spectrum showed that the methylation was complete. Similarly, the nmr



Scheme I: Synthesis of 1-(4-Biphenylyl)-n-pentyl Hydrogen 3-Methyl-3-methoxyglutarate.

spectrum showed no hydroxyl proton; instead, a sharp singlet attributed to the methoxyl protons was observed at 3.20 ppm.

Ozonolysis of XV followed by very careful oxidative destruction of the ozonide afforded 3-methyl-3-methoxyglutaric acid (XVI) in a yield of 75%. This compound was the key intermediate of the entire sequence of reactions. Presence of the acid was clearly indicated by the severely broadened O-H stretching band in the 2500-2700 cm^{-1} region of the infrared spectrum and by the carbonyl peak at 1710 cm^{-1}. The structure of the diacid was proved by the nmr observation of two exchangeable protons at 9.93 ppm as well as other peaks described above for corresponding portions of the molecule.

Several methods of esterification of XVI with 1-(4-biphenylyl)-n-pentanol (XVII) were examined before the desired half ester-half acid was obtained. Initially it was thought that the ester could be obtained by opening anhydride XLII with the alcohol using the method of Palazzo et al. (162). This route exhibited two deficiencies: first, dehydration of the diacid XVI provided the anhydride XLII as a heavy oil which resisted both spectral characterization and purification. Furthermore, it appeared that this compound was not stable under normal conditions and even attempts to esterify the crude anhydride were unsuccessful. Second, Palazzo's method of esterification requires harsh conditions for extended periods (heating

at reflux in pyridine for 8 hours).

The esterification method of Steglich and Höffle (175) appeared promising since it takes place under mild conditions with catalysis by 4-dimethylaminopyridine. However, this method also requires the anhydride. Perhaps because of the necessity of using crude anhydride, this reaction afforded very low yields (5%) of impure acid-ester V.

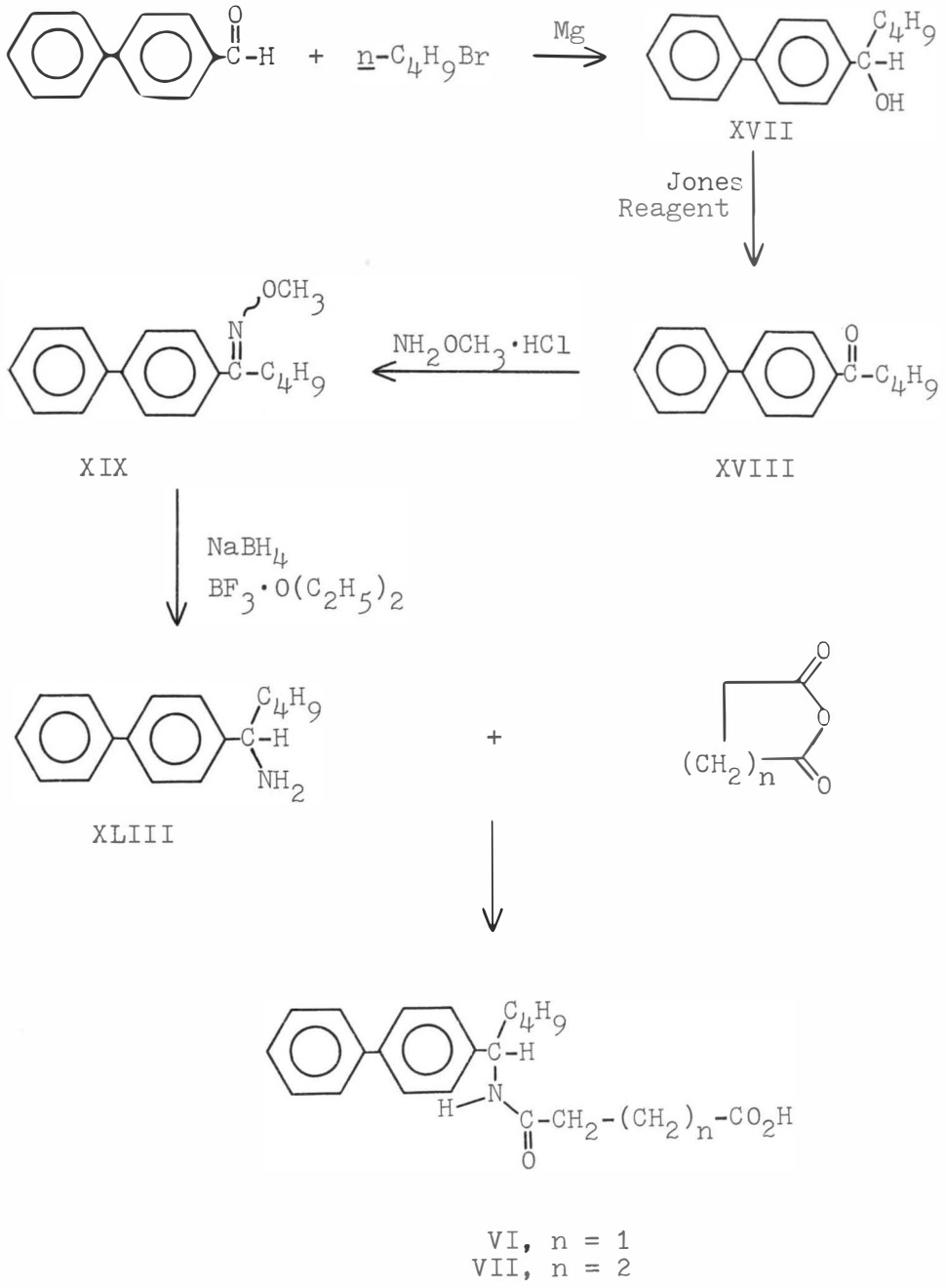
The method of Büchi et al. (167) was found to provide the desired ester V in acceptable yields. The problem of using impure anhydride was surmounted with this technique because the diacid XVI is the substrate for esterification. Even though a statistical yield (50%) of the desired monoester V was obtained, this reaction was superior to the other esterification methods available.

This acid-ester was spectrally characterized by the broadened infrared absorption in the 2500-2800 cm^{-1} region indicating the free carboxylic acid. Additionally, both ester (1740 cm^{-1}) and carboxylic acid (1713 cm^{-1}) carbonyl bands were observed. A significant aspect of the nmr spectrum was the internal consistency of integral ratios between the aromatic absorption at 7.00-7.79 ppm and both the absorptions due to the methoxyl and methyl protons at 3.15 and 2.72 ppm, respectively. Since the latter two functional groups were derived from a different reagent than the aromatic protons, this self-consistency indicates that the desired monoester was obtained in good purity.

The preparation of N-[1-(4-Biphenylyl)-n-pentyl]

Succinamic (VI) and Glutaramic (VII) acids is described below. The synthetic sequence followed for the preparation of these amides is shown in Scheme II. Oxidation of alcohol XVII led smoothly to the corresponding ketone XVIII in excellent yields (80-90%). This compound was spectrally characterized by the absence of hydroxyl absorption and the presence of a strong ketone band (1669 cm^{-1}) in the infrared as well as by loss of signals for the exchangeable proton as observed in the nmr spectrum. Similarly, conversion of ketone XVIII to the methoxylimine XIX by the method of Feuer and Braunstein (171) took place smoothly and in good yield (90%). This conversion was readily followed with infrared spectra by the loss of the ketone carbonyl band and the appearance of absorption at 1610 cm^{-1} in the infrared spectrum due to C=N stretching vibrations. Appearance of a very sharp singlet at 3.97 ppm in the nmr spectrum ($-OCH_3$) also indicated presence of the desired imine. Reduction of this compound by the method of Boots et al. (172) proceeded as expected; however, the major difficulty in this series of reactions was purification and characterization of the free amine XLIII.

The free amine XLIII was initially obtained as a thick and impure oil or semi-solid and spectral characterization of this material was not conclusive. This problem was solved by a minor modification of the work-up procedure. Rather than isolating the free amine as a semi-solid, the dry solution of suspected free amine was treated with



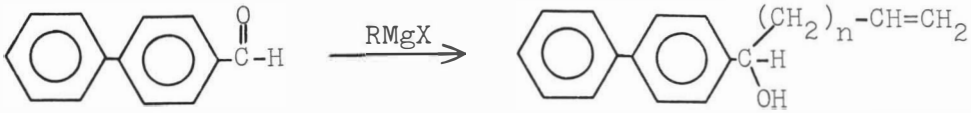
Scheme II: Synthetic sequence for the preparation of succinamides and glutaramides.

ethereal hydrogen chloride (195). This afforded a precipitate of the amine hydrochloride XX which was easily purified in a yield of 89% and characterized both spectrally and by elemental analysis.

Conversion of the amine to its hydrochloride salt XX to facilitate handling required slight modification of the subsequent preparation of the amides VI and VII. Thus, prior to each amidation reaction a slight excess of amine hydrochloride was treated with dilute base, then extracted into the solvent to be used for amide formation and dried. The use of this solution of the amine XLIII greatly aided in the preparation of amides VI and VII in high yields (77-84%).

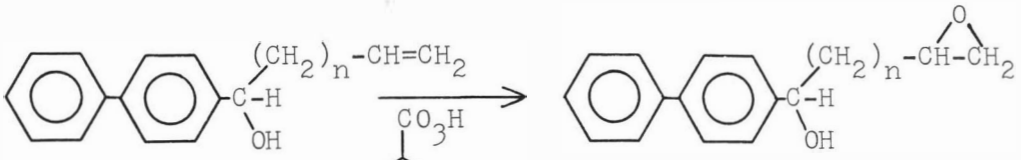
The amides were readily characterized by infrared spectroscopy by the N-H vibration in the 3250 cm^{-1} region as well as by a pair of carbonyl bands due to the free carboxylic acid and the amide carbonyl vibrations. The nmr spectrum of these compounds showed a pair of protons as broad singlets (-CONH and -COOH) downfield from the aromatic absorption as well as the characteristic methine absorption in the 5.0 ppm range.

The synthesis of 1-(4-biphenylyl)-alkenyl (VIIIa-f) and epoxyalkyl (IXa-f) hydrogen succinates and hydrogen glutarates is described below. The general approach to the synthesis of these acid esters is outlined in Scheme III. The Grignard reagents derived from ω-bromo-1-alkenes reacted smoothly with 4-biphenylcarboxaldehyde (XLIV) to



XLIV

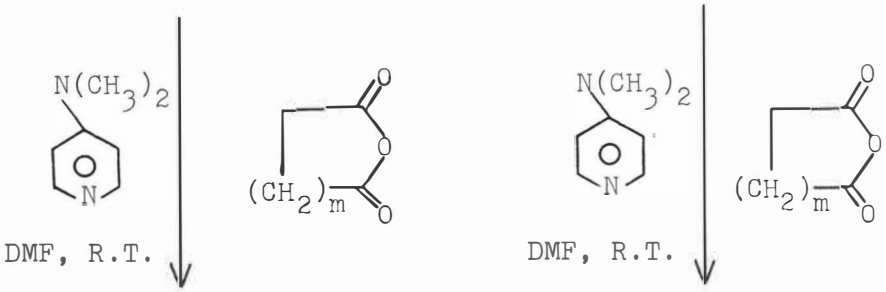
XXII, n = 1
XXIII, n = 2
XXIV, n = 3

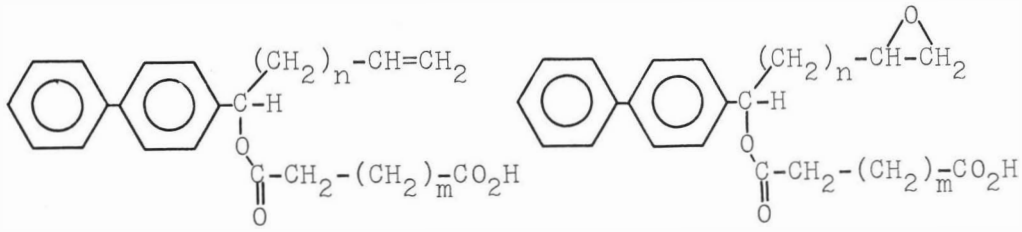


XXII-XXIV

$CHCl_3$, R.T.

XXV, n = 1
XXVI, n = 2
XXVII, n = 3





VIIIa, n = 1; m = 1
b, n = 1; m = 2
c, n = 2; m = 1
d, n = 2; m = 2
e, n = 3; m = 1
f, n = 3; m = 2

IXa, n = 1; m = 1
b, n = 1; m = 2
c, n = 2; m = 1
d, n = 2; m = 2
e, n = 3; m = 1
f, n = 3; m = 2

Scheme III: The synthesis of 1-(4-biphenylyl)-alkenyl and epoxyalkyl hydrogen succinates and hydrogen glutarates.

provide the alcohols XXII-XXIV in 70-90% yields. Special precautions were necessary only for the synthesis of alcohol XXII because of the highly reactive nature of the allyl Grignard reagent. The double addition method of Tschesche and Machleidt (165) was used in order to maximize the yield of the desired alcohol and minimize the occurrence of coupling of the Grignard reagent, the major side reaction in this system.

Alcohols XXII-XXIV have several common spectral features which were used to structurally identify each member of the series. The infrared spectrum was used only as an indicator of the presence of the alcohol functional group. Absorption bands used for this purpose were those derived from the free O-H (3500-3600 cm^{-1}) and hydrogen bonded O-H (3400-3500 cm^{-1}) vibrations. Proof of structure was accomplished with nmr spectrometry. Several of the maxima exhibited by these alcohols became important features of the spectra of subsequent compounds. The vinyl group of XXII-XXIV was observed as a very complex series of multiplets at approximately 5.5-6.1 ppm (-CH=) and as a second set of multiplets at 4.8-5.2 ppm ($=CH_2$). Only in the case of XXII was the methylene group located β to the aromatic ring observed as a separate maximum. In the other cases these protons were either partially superimposed upon or obscured by absorption due to the remaining methylene groups. Finally, the methine proton α to the aromatic rings was diagnostic of alcohols XXII-XXIV as well

as subsequent compounds and consistently appeared as a well defined triplet at approximately 4.6 ppm.

As with the 3-methyl-3-methoxyglutaric acid ester V discussed above, a number of methods were investigated for conversion of the 1-(4-biphenylyl)-alkenyl alcohols XXII-XXIV to their respective hydrogen succinates and hydrogen glutarates before a satisfactory procedure was found. Since several of the desired acid-esters are polyfunctional systems bearing delicate functional groups, one of the major criteria for the choice of an esterification method was the necessity to avoid strenuous reaction conditions. Thus, the method of Palazzo et al. (162) was unacceptable due to the extended reaction times and elevated temperatures involved. Alternatively, the method of Büchi et al. (167) provided a gentle method of esterification; however, this method requires the acceptance of only a statistical yield (50%) of monoester from a dicarboxylic acid.

With respect to vigor of reaction conditions and available yields, the esterification method of Steglich and Höffle (175) provided an acceptable means of synthesis of the desired half acid esters. The reaction, catalyzed by 4-dimethylaminopyridine, takes place entirely at room temperature and has provided satisfactory yields of both the alkenyl (40-80%) and epoxyalkyl (30-70%) hydrogen succinates and hydrogen glutarates. In their original procedure Steglich and Höffle employed an excess of

triethylamine to bind the free carboxylic acid as it was formed. However, examination of the reaction conditions showed that both the integrity of the product and the acceptable yield of the reaction could be maintained when this reagent was omitted from the reaction solution. Therefore, each of the esters illustrated in Scheme III have been prepared using this modification of the original procedure.

Infrared spectra of esters VIIIa-f and IXa-f indicated that the desired acid-esters had been obtained. Broadening in the 3100-3300 cm^{-1} region and the medium very broad absorption observed at 2500-2700 cm^{-1} is diagnostic of the free carboxylic acid portion of these compounds. Additionally, for each of these esters a pair of carbonyl peaks was observed, the one at higher wavenumber being due to the ester carbonyl while the lower wavenumber peak is attributable to carboxylic acid carbonyl vibration. Absorption due to the epoxide ring, as discussed below for epoxyalcohols XXV-XXVII, was also observed.

Structural information obtained from the nmr spectra of VIIIa-f includes the maxima characteristic of the vinyl group as previously discussed for the alkenyl alcohols. Similarly, both the alkenyl esters VIIIa-f and the epoxyalkyl esters IXa-f exhibited maxima described above for the methine proton α to the aromatic rings. In every case, the succinate esters showed a very sharp singlet caused by the four succinate methylene protons. Self-consistency of the ratio of the integral of this peak to

that of the aromatic absorption was an important criterion of purity since the respective peaks originated from different reagents. This was not always possible with the hydrogen glutarates because the glutarate methylene protons were observed as a complex set of multiplets which overlapped with the peaks from the methylene protons of the alkenyl or epoxyalkyl side chain.

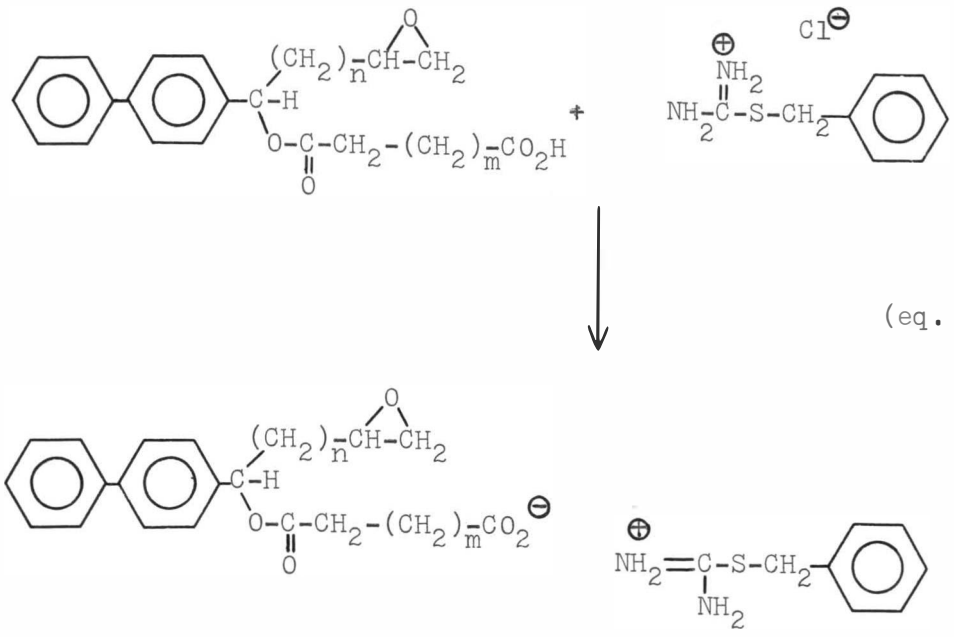
Epoxidation of the alkenyl alcohols was accomplished using the technique described by Fieser (176) which provided mild reaction conditions. Reaction of alkenyl alcohols XXII-XXIV with m-chloroperbenzoic acid in chloroform at and below room temperature provided the desired 1-(4-biphenylyl)-epoxyalkyl alcohols XXV-XXVII in yields of 92-99%. Conversion of alkenes XXII-XXIV to the corresponding epoxides XXV-XXVII was readily followed with infrared spectra by the appearance of the characteristic epoxide peaks around 840, 910, and 1260 cm^{-1}. Nuclear magnetic resonance spectra of these compounds showed a very clear loss of absorption due to the vinyl protons and the appearance of complex multiplets due to epoxide ring protons in the region 1.5-3.0 ppm. The triplet due to the methine proton α to the aromatic rings continued to be a useful diagnostic signal.

Placement of the epoxidation reaction in the complete synthetic design is worthy of note. It may appear from Scheme III that the epoxidation of alcohols XXII-XXIV could conceivably be performed either before or after esterification

with equal success. A co-product of the epoxidation reaction is m-chlorobenzoic acid which is separated from the crude epoxide as its sodium salt. This is accomplished by treatment with mild aqueous base. If the epoxidation were performed subsequent to esterification, a mixture of the sodium salts of m-chlorobenzoic acid and the desired hydrogen succinate or hydrogen glutarate would be obtained, providing a rather difficult separation problem. For this reason, the epoxidation reaction must be performed only prior to esterification. In this instance, the sequence of reactions means the difference between obtaining the desired compounds fairly pure and in good yield or an inseparable mixture.

The alkenyl esters VIIIa-f and the epoxyalkyl esters IXa-f exist under standard conditions as clear, viscous oils. To facilitate elemental analysis of these compounds they were converted to the corresponding S-benzylthiuronium salts by the method of Donleavy (168) as modified by Boots et al. (158) as illustrated in equation 6. These salts are moderately high melting solids which may be purified by recrystallization. Since they were used only for elemental analysis, infrared and nmr spectra were not determined for these salts.

Elemental analysis of these salts provided an interesting problem. Several S-benzylthiuronium salts which had been prepared from esters whose structure and purity had been proved by infrared and nuclear magnetic resonance



(eq. 6)

techniques consistently exhibited carbon analysis 1.0-1.5 percent below theory while nitrogen and hydrogen analyses were within the acceptable range (± 0.4%). The explanation and solution to this formidable problem lay in the fact that the S-benzylthiuronium salts were initially precipitated from aqueous solution before they were recrystallized. The crude salts appear to have precipitated as a complex with one-half mole of water associated with each mole of salt. Thus, inclusion of one-half mole of water in the calculation of theoretical elemental composition gave values for percent carbon which were consistently 1.0-1.5% lower than the theoretical carbon percent calculated without inclusion of water. The inclusion of

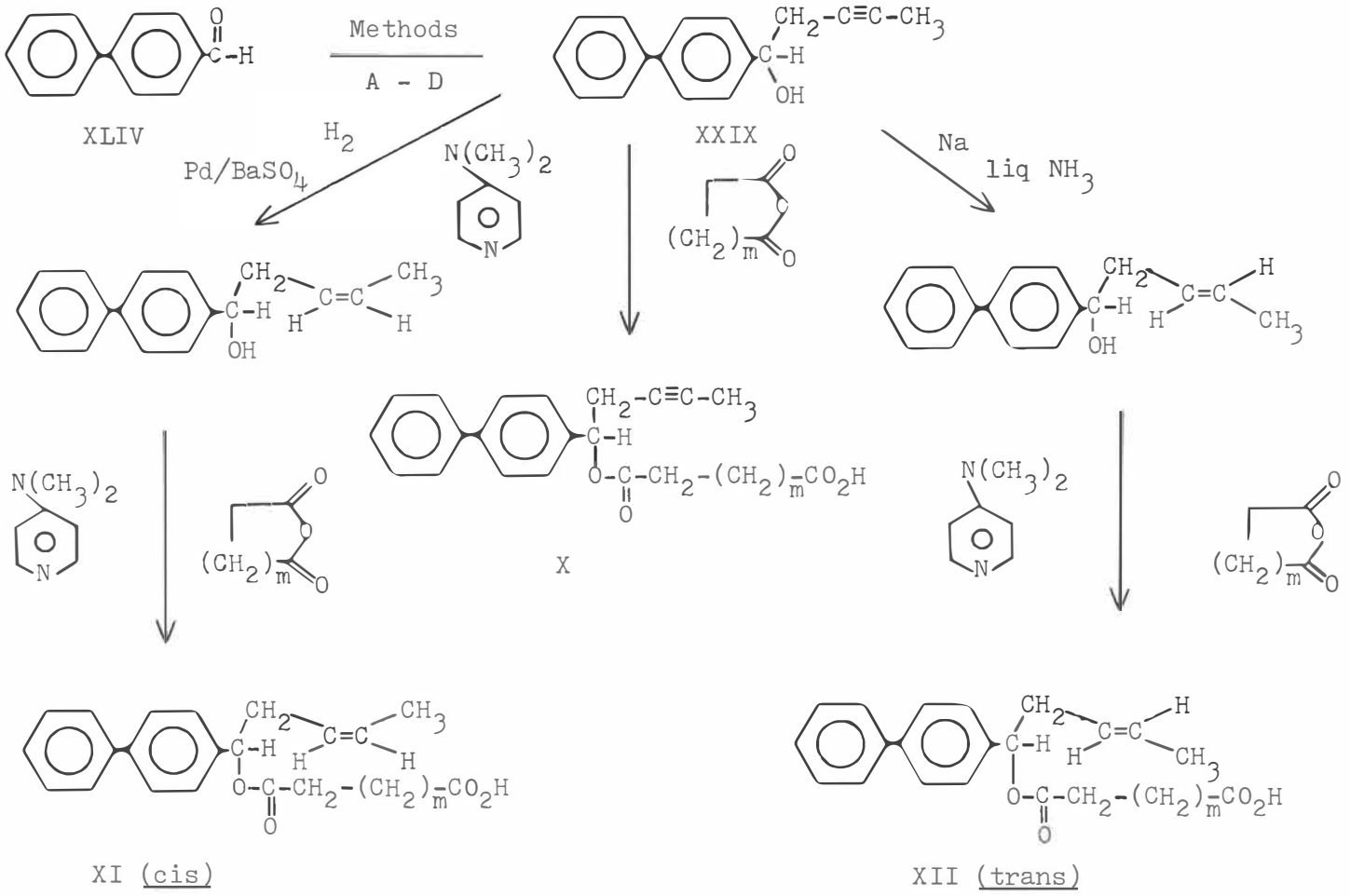
one-half mole of water in the calculation of theoretical nitrogen and hydrogen percent gave values which differed from those calculated without inclusion of water by an amount less than the experimental error of measurement, that is, within approximately ± 0.25% of theory calculated without water. In two instances the association of one mole of water per mole of salt was observed.

Proof of this hypothesis required removal of the water and subsequent analytical results showing agreement with theoretical percentages. A single salt, the S-benzylthiuronium salt of 1-(4-biphenylyl)-3-butenyl hydrogen succinate (VIIIa-S) had a melting point high enough to withstand the drying procedures required to remove the firmly associated water. If the drying period was too short not all of the water was removed and if too long, the salt decomposed. Drying the above salt for 8.5 hours at 110° under 1 mm Hg pressure afforded the truly dry salt whose elemental analysis fell within experimental error of the calculated values. Further support for this hypothesis and its proof is the fact that as drying conditions became more vigorous (that is, progressively increasing time and temperature of drying from short periods at room temperature to the final conditions) the percent carbon determined slowly increased until it was within experimental error of theory when dried under the final conditions.

The attempted synthesis of alkynyl alcohol XXIX for the study of geometrical isomerism in the ester side chain

is described below. Each agent described above contains the double bond or epoxide functional group in a terminal position of the alkyl moiety where geometric isomerism is not possible. Therefore, it was of great interest to determine the effect of geometrical isomers in the alkyl group on the activity of these agents. This would provide very useful information not only about the structural requirements for efficient inhibition of rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase but also concerning the structure of the enzyme itself in the region where these inhibitors bind. The study of these effects requires the synthesis of internal alkenyl hydrogen succinates and hydrogen glutarates and the proposed synthetic sequence to obtain these compounds is outlined in Scheme IV. It should be noted that this sequence of reactions provides a means of obtaining not only the cis and trans alkenyl esters (XI and XII, respectively) but also the alkynyl esters X which are structurally locked into linearity in the region of interest. The key intermediate in this proposed synthetic sequence is the alkynyl alcohol XXIX, and several methods were examined in the attempted synthesis of this compound.

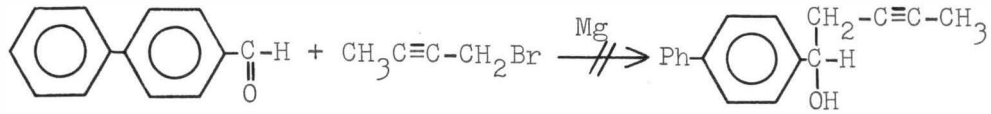
The first attempt to prepare alkynyl alcohol XXIX was by the Grignard reaction (equation 8). Using the method of Sharifkanov and Skhmedova (177), 1-bromo-2-butyne (XXVIII) was prepared (equation 7). The presence of this compound was readily demonstrated by infrared absorption at 2235 cm^{-1}



Scheme IV: Proposed synthetic sequence for geometric isomers in alkyl moiety.

$$CH_3\text{-}C{\equiv}C\text{-}CH_2OH + PBr_3 \longrightarrow CH_3\text{-}C{\equiv}C\text{-}CH_2Br \qquad \text{(eq. 7)}$$

XXVIII



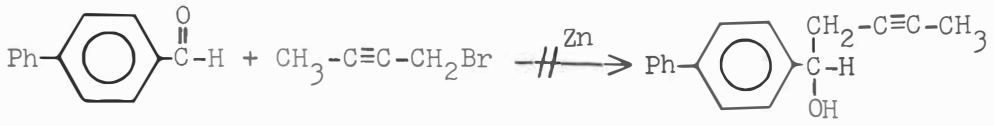
XXVIII

XLIV XXIX (eq. 8)

caused by alkyne stretching vibrations. The nmr spectrum exhibited the expected quartet and triplet (methylene and methyl protons, respectively) due to coupling across the triple bond.

The reaction of 1-bromo-2-butyne (XXVIII) with 4-biphenylcarboxaldehyde (XLIV) in the normal Grignard manner afforded in every case unchanged starting aldehyde. The double addition technique of Tschesche and Machleidt (165) for highly reactive Grignard reagents and the use of concentrated solutions did not provide any improvement. Failure of the reaction to take place was demonstrated by the identical infrared spectra of the recovered material and authentic 4-biphenylcarboxaldehyde.

An alternate route to alcohol XXIX was *via* a zinc complex as described in the work of Henbest *et al.* (178). These workers were able to carry out Reformatsky-type reactions with propargyl bromides; however, it was not



XLIV XXVIII XXIX (eq. 9)

possible to successfully apply their techniques to the present work (equation 9). Several variations of the reaction conditions were made and in each case an allene was obtained rather than the desired alkynyl alcohol.

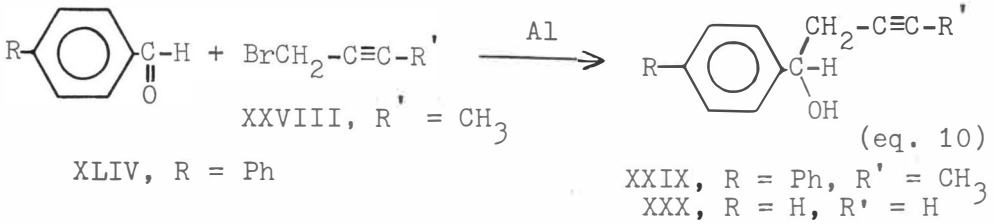
The allene was detected by absence of alkyne absorption at 2100-2300 cm^{-1} and the presence of a sharp peak at 1963 cm^{-1} in the infrared spectrum. The nmr spectrum of the material obtained from the initial attempt to prepare the alkynyl alcohol by this method was inconclusive. On subsequent attempts if the infrared spectrum indicated the presence of an undesired allene the nmr spectrum was not recorded.

Modifications made in the above procedure may be briefly described as follows: the crude ether extract of the ice-10% acetic acid slurry (see experimental section) was washed with copious quantities of water rather than with mild base. This was an attempt to avoid base catalyzed formation of the allene in the work-up procedure. The activity of the zinc surface was varied by altering the etching time up to 40 minutes. To guard against thermal rearrangement, the reaction was conducted at room temperature with the addition of reactants being carried out at ice

bath temperature. This experiment and one performed entirely at -78° were fruitless. In addition, discontinuation of the use of mercuric chloride as an initiator and conducting the reaction in dilute solution afforded products which were not well defined. Each of these modifications of the reaction conditions gave a clear, yellow liquid, the infrared spectrum of which consistently exhibited a sharp, medium band at 1962-1965 cm^{-1}, indicating the presence of an allene, and a complete lack of absorption in the region 2100-2300 cm^{-1}, indicating the absence of an alkyne.

Several of the reactions attempted in the present investigation were initially tested on a model system in order to establish and refine the reaction conditions prior to experiments with 4-biphenylcarboxaldehyde (XLIV). The model chosen was benzaldehyde and all structure determinations were made on the basis of infrared and nmr data. In general, elemental analyses were not determined for model systems.

An attractive alternative to the Reformatsky-type reaction was the work of Lauger et al. (181) who prepared propynyl esters. These workers recognized the problem of allene formation and claimed that by employing an aluminum complex rather than magnesium or zinc the alkyne could be formed in high yields while formation of the allene was essentially negligible. Using benzaldehyde and 1-bromo-2-propyne as a model system provided encouraging results;



XXVIII, R' = CH_3

XLIV, R = Ph

(eq. 10)

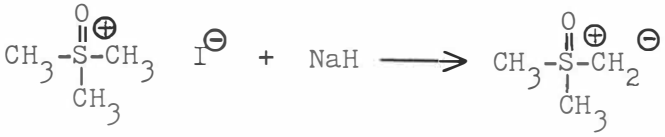
XXIX, R = Ph, R' = CH_3
XXX, R = H, R' = H

the infrared spectrum of the product exhibited both alkyne and alcohol bands and the nmr spectrum provided structural proof of the model alkynyl alcohol (equation 10, R = H, R' = H). Unfortunately, when the same reaction was attempted using the biphenyl and the 2-butynyl system (equation 10, R = Ph, R' = CH_3) only the starting aldehyde was recovered.

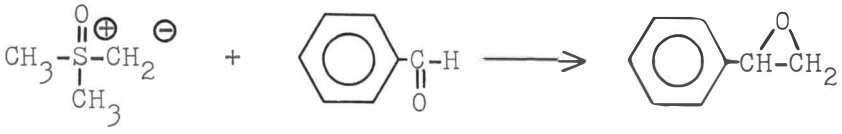
Failure of the aluminum method to provide the desired alkynyl alcohol led to attempts to open an aryl substituted epoxide as a means of synthesizing alcohol XXIX. Extensive use of the benzaldehyde model system was employed in this series of reactions. The model epoxide XXXII was prepared by the method of Corey and Chaykovsky (183) via the ylide derived from trimethylsulfoxonium iodide (XXXI) (182), as shown in equations 11-13. This elegant conversion of an aldehyde to an epoxide had a single drawback: the yield of epoxide obtained in this investigation was very low.

The infrared spectrum of the epoxide showed the expected characteristic peaks due to epoxide C-O vibrations. It is interesting to note that in the nmr spectrum of both the phenyl and the biphenylyl epoxides each of the three aliphatic protons is observed as a pair of doublets. In

XXXI (eq. 11)



XXXI (eq. 12)



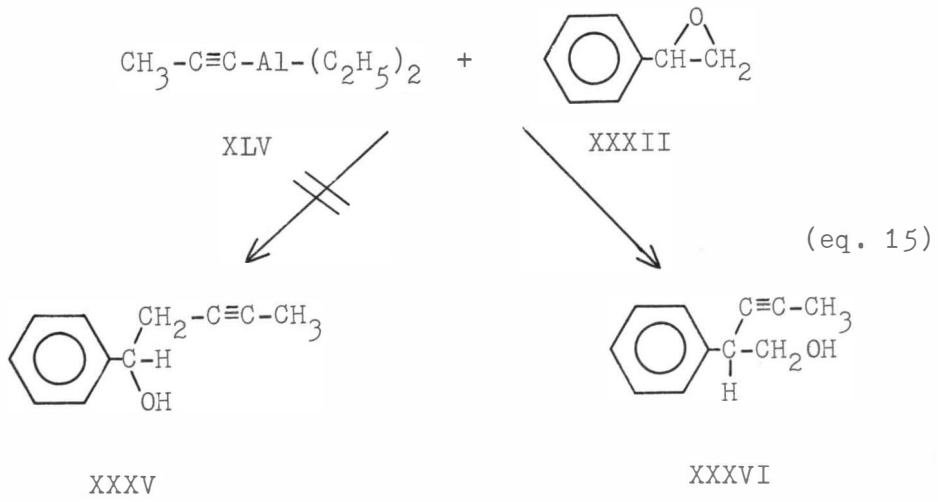
XLIV XXXII (eq. 13)

the case of the proton α to the aromatic rings the multiplicity arises from spin-spin splitting by both β protons. The β protons are split into doublets by the α proton, and the doublets from each β proton are further split into a pair of doublets by the other β proton, a case of geminal spin-spin interactions.

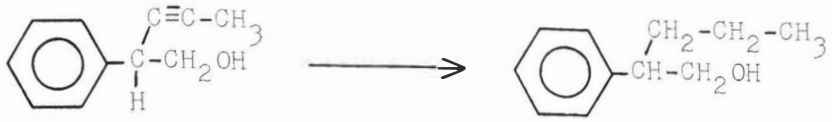
It was initially thought that the epoxide could be opened in the desired direction by means of diethylpropynyl aluminum (XLV) prepared (equation 14) by the method of Nagata and Yoshioka (185). This reagent was used in an

XXXIV XLV (eq. 14)

attempt to open the model epoxide (equation 15) by the method of Fried et al. (186). Surprisingly, the desired 1-phenyl-3-pentyn-1-ol (XXXV) was not obtained; the nmr spectrum indicated that the epoxide may have opened in the unanticipated direction to give the primary alcohol XXXVI. If XXXV had been obtained, the nmr spectrum would be expected to show the methyl group as a triplet, the methylene protons as a complex multiplet, and the methine proton as the usual triplet. The spectrum of the material obtained was clearly not that of the desired alcohol XXXV since it exhibited a doublet integrating for the same number of protons as a sharp signal superimposed upon a complex band. These spectral features can be accounted for by the primary alcohol XXXVI.



Catalytic reduction (equation 16) of the suspected 2-phenyl-3-pentyn-1-ol (XXXVI) was shown by nmr data to



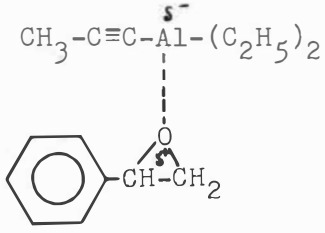
(eq. 16)

XXXVI XXXVII

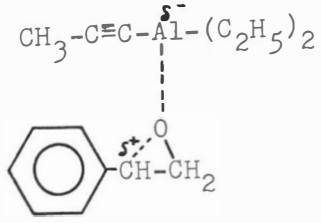
have produced the corresponding alkane 2-phenyl-1-pentanol (XXXVII). This conversion was followed by the appearance of broad complex multiplets at 0.6-1.8 ppm ($-CH_2-CH_2-CH_3$), a sharp doublet at 3.68 ppm ($-CH_2-O-$), and the single methine proton as a multiplet centered on 2.74 ppm. The nmr spectrum was proof that saturated alcohol XXXVII was obtained from reduction of XXXVI and therefore, XXXVI must have been 2-phenyl- rather than the expected 1-phenyl-3-pentyn-1-ol (XXXV). These results are similar to those of Boireau et al. (198) who studied the reaction of trimethylaluminum with 3-phenyl-1,2-epoxypropane.

Primary alcohol XXXVII is the single case of a model system where elemental analysis data were determined and these data show that the product from the catalytic reduction of alcohol XXXVI fit the theoretical elemental composition of 2-phenyl-1-pentanol. The nmr data for alcohol XXXVI, the spectral structure proof of alcohol XXXVII and the analytical data for this compound conclusively show that the propynyl alane reagent caused the epoxide to open in the S_N1 direction rather than in the anticipated S_N2 direction.

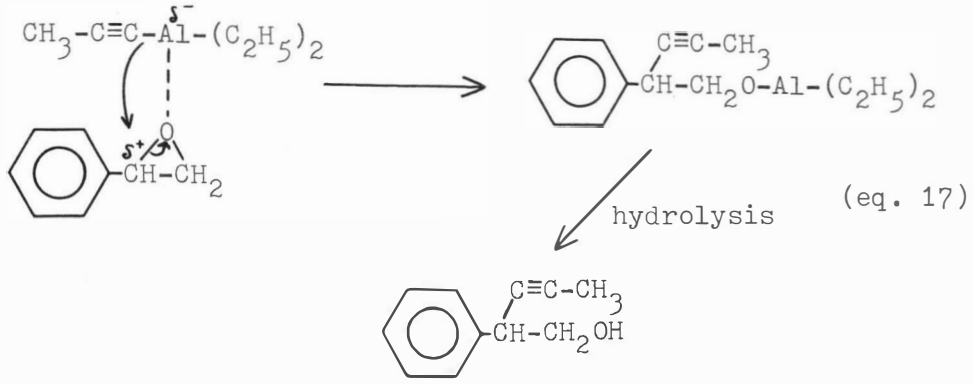
It is tempting to speculate on the mechanistic implications of these results. Apparently, the aluminum atom of the alane reagent is a strong enough Lewis acid to preferentially associate with the oxygen atom of the epoxide. This would weaken the two carbon-oxygen bonds and cause some electron deficiency in the epoxide ring (XLVI). Since the α carbon atom is capable of forming a relatively stable carbonium ion by virtue of resonance delocalization of the charge into the benzene ring it is reasonable to suggest that structure XLVIa provides a great contribution to the true electronic structure of the suggested alane-epoxide complex. If this is indeed the case, then it is plausible that the alane reagent, by virtue of its association with the oxygen atom of the epoxide, delivers the propynyl group exclusively to the α rather than to the β carbon atom of the epoxide (equation 17). This agrees with the mechanistic proposal of Lundeen and Oehlschlager (199) who have studied the reaction of triethylaluminum with 1,2-epoxypropane. These investigators have observed evidence that a second alane molecule participates in opening the epoxide.



XLVI



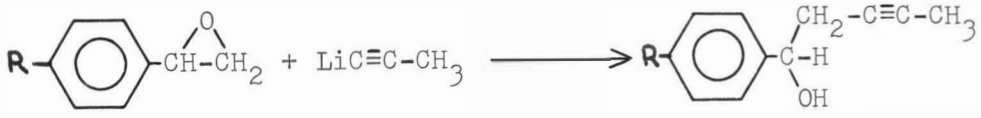
XLVIa



(eq. 17)

XXXVI

In light of these interesting but unproductive results with the alane reagent, attempts were made to open the model system (equation 18, R = H) with propynyllithium. For the model epoxide this reaction gave moderately low yields (40-50%) of the desired alkynyl alcohol XXXV and exhibited a poor material balance. Infrared and nmr spectra of XXXV provided clear proof that the anticipated product had been obtained. When the same reaction was attempted with the biphenylyl epoxide XXXIII (equation 18, R = Ph) yields and material balance became even worse than for the model system.



(eq. 18)

XXXII, R = H
XXXIII, R = Ph

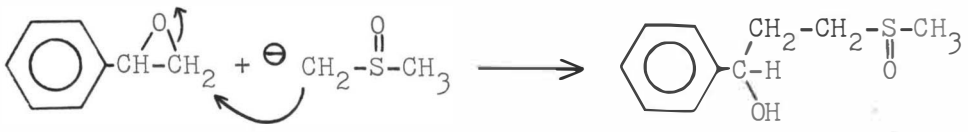
XXXV, R = H
XXIX, R = Ph

Since the solvent used for this reaction was dimethylsulfoxide it was considered possible that the desired product was not sufficiently soluble in the extraction solvent (ether) to be efficiently removed from the reaction mixture during the work-up procedure. Testing this possibility, it was found that the desired alkynyl alcohol was indeed sufficiently soluble in ether to have been removed from the reaction mixture. In addition, using known alkynyl alcohol, the work-up procedure was scaled down by a factor of fifty and the entire work-up was carried out. In this experiment 8.0 mg of a 9.4 mg sample of alkynyl alcohol XXIX survived the procedure. This indicated that the low yeild and material balance of this reaction was not caused by a preferential solubility of the product in the dimethylsulfoxide-water phase.

A possible but untested explanation for the low yields and poor material balance of alcohols XXXV and XXIX is that propynyllithium may have participated in an exchange reaction with the solvent, generating the dimsyl carbanion (XLVII, equation 19). The presence of this carbanion in

$$LiC{\equiv}C\text{-}CH_3 + CH_3\text{-}\overset{\overset{O}{\|}}{S}\text{-}CH_3 \longrightarrow \underset{\text{XLVII}}{CH_3\text{-}\overset{\overset{O}{\|}}{S}\text{-}CH_2^{\ominus}} \; Li^{\oplus} + HC{\equiv}C\text{-}CH_3 \qquad \text{(eq. 19)}$$

the reaction mixture would provide a nucleophile capable of opening the epoxide (equation 20) to give a polar product



(eq. 20)

which could have been trapped in the dimethylsulfoxide-water phase. However, as noted above, this possibility is entirely speculative and untested.

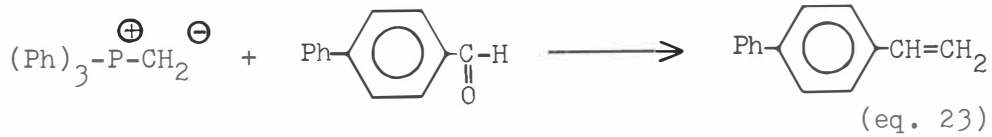
Because preparation of biphenylyl epoxide XXXIII was a very low yield reaction, it was decided to employ an alternate route to epoxide preparation. The route chosen is shown in equations 21-24 and utilized the Wittig reaction (189, 190).

(eq. 21)

XXXVIII

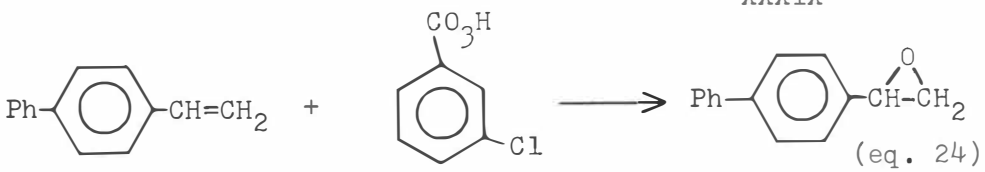
(eq. 22)

XXXVIII



(eq. 23)

XXXIX



(eq. 24)

XXXIX

XXXIII

Both formation of (4-biphenylyl)ethene (XXXIX), (equation 23) and its subsequent epoxidation product XXXIII (equation 24) took place smoothly and provided the desired products in acceptable yields (45 and 50%, respectively). Alkene XXXIX exhibits an nmr spectrum which is similar to and lends itself to the same analysis as the spectrum of epoxides XXXII and XXXIII discussed above. Unfortunately, opening of this epoxide by the method of Fries (188) as illustrated in equation 18 failed to provide the necessary key intermediate alkynyl alcohol XXIX in sufficient quantities to continue the study of geometrical isomers derived from this compound. Therefore, attempts to complete this study were postponed.

The homologous butynyl system XL also lends itself to study of geometrical isomers derived from it. Using a slight modification of the work of Smith and Kuehn (192) 1-(4-biphenylyl)-2-butyn-1-ol (XL) was prepared (equation 25) by addition of propynyllithium to the carbonyl group of 4-biphenylcarboxaldehyde. The conditions of this reaction are very delicate and time is a critical factor. The expected doublet and quartet of the methyl and methine protons respectively were clearly observed in the nmr spectrum of XL. It is important to note that since propynyllithium will add to the carbonyl group of 4-biphenylcarboxaldehyde, perhaps minor modifications of the reaction conditions will provide a means of opening the previously discussed epoxides in the desired manner.

(eq. 25)

XL

Finally, esterification of alkynol XL with succinic anhydride has been accomplished by the method of Steglich and Hoffle (175) to give the corresponding monoester XLI in 31% yield. This ester shows the expected spectral characteristics similar to the previously described esters and to the parent alcohol XL. This compound provides the entrance to a geometrical isomer study similar to that depicted in Scheme IV.

BIOLOGICAL RESULTS

Hepatic microsomes were obtained from male Sprague-Dawley rats by the method described in the experimental section. In order to maximize HMG CoA reductase content in these preparations, the livers were harvested between 12:05 and 12:25 AM during the peak of the diurnal rhythm. In spite of this precaution, the enzyme activity of different preparations was found to vary. Shefer et al. (197) have observed that the specific activity of HMG CoA reductase is highest in young animals and decreases with increasing age; this decrease is more pronounced in females than in males. The use of young male rats with a mass of approximately 100 g as the source of microsomes provided preparations with fairly consistent activity of the enzyme among different preparations.

An additional method was employed to ensure that the inhibitory activity of all compounds could be compared even though they were tested with several different microsomal preparations. A previously prepared compound with known inhibitory properties, 1-(4-biphenylyl)-n-pentyl hydrogen succinate, (III, m = 2) was tested concurrently with all other inhibitors. This allowed comparison of all inhibitors to the standard III (m = 2) and therefore also allowed valid comparisons to be made among all the inhibitors.

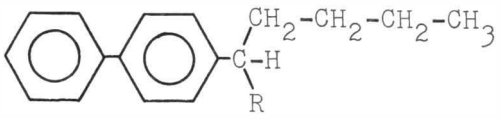
The unit used to express inhibitory activity is the ratio of inhibitor concentration to substrate concentration

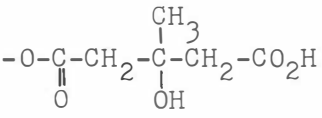
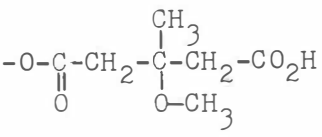
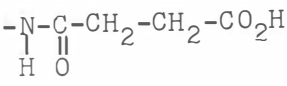
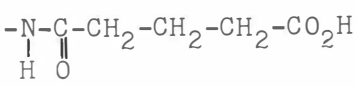
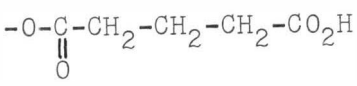
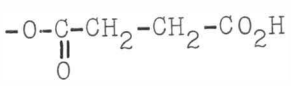
which results in 50% inhibition of the conversion of HMG CoA to mevalonic acid. This unit is expressed as $(I/S)_{0.5}$ and is determined by varying the inhibitor concentration in the test system. In some cases the solubility of the inhibitor limits the degree of inhibition and a reduction of 50% cannot be obtained. When this occurs, the degree of inhibition at the stated inhibitor concentration is specified.

Table I lists the inhibitory activity of the 3-methyl-3-methoxy substrate analog V as well as the 3-methyl-3-hydroxy derivative IV for comparison. The fact that the 3-methoxy compound V is elevenfold less active than the corresponding 3-hydroxy analog IV may be interpreted in two ways. The 3-methyl-3-hydroxy analog IV may bind to the enzyme in such a manner that the 3-hydroxy group is located in a pocket or other confining structure formed by the enzyme surface. If this area is small enough to accomodate only the hydroxyl group then ether V may be too large to bind in a manner analogous to the normal binding of the 3-hydroxyl group. If true, this could slightly alter the binding position of other parts of the inhibitor and the observed loss of activity for V would be caused by the O-methyl group blocking the proper approach of the inhibitor to the binding subsites.

A more reasonable explanation of the loss of activity seen with V is based on a direct binding function of the 3-hydroxy group. As previously discussed, this group could participate in binding by acting as a hydrogen donor

Table I: Inhibition of Rat Liver Microsomal HMG CoA Reductase by



Compound Number	R	$(I/S)_{0.5}$
IV		1
V		11
VI		30 (18% inhibition)
VII		20
----		11
III (m = 2) (Reference Standard)		11

(through the hydroxyl hydrogen) or as a hydrogen acceptor (through the hydroxyl oxygen) in hydrogen bonding with the enzyme. Replacement of the hydroxyl hydrogen of IV with the methyl group of V destroys only the ability of the inhibitor to act as hydrogen donor, not its ability to act as acceptor. Therefore, it can be concluded that the loss of activity exhibited by V with respect to IV is caused by its inability to participate in hydrogen bonding with the enzyme. The demonstration of hydrogen bonding in these compounds also clearly shows that a hydrogen bond acceptor is located on the enzyme surface at the binding site. This is a valuable binding aid and its presence may be exploited by future inhibitors to enhance reversible binding.

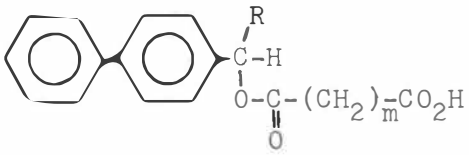
The inhibition of HMG CoA reductase caused by the amides VI and VII is shown in Table I. As can be seen, the succinamide exhibited poor activity while the corresponding glutaramide was approximately one-half as active as the reference ester III (m = 2). Previous discussion showed that the ester functional group is not necessary for binding. However, it should be noted that the compounds examined to prove this contained either no substituent capable of hydrogen bonding in the ester region (carbon analog) or groups capable only of acting as hydrogen acceptor (ether and ester analogs). Therefore, these experiments not only showed that the ester group is not required for binding, but also that the surface of the enzyme probably does not contain a hydrogen donor for

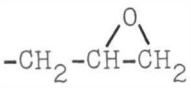
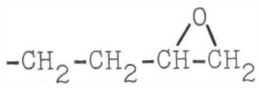
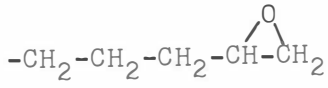
hydrogen bonding in proximity to the ester function when it is bound. It is possible that a hydrogen acceptor for hydrogen bonding is located on the enzyme close to the ester group of the inhibitors. If this were true, it would not participate in the binding of inhibitors which contained no hydrogen donor, such as the esters, ether and carbon analogs. Amides VI and VII represent replacement of the ester function with a group which is similar to the ester group in steric and electronic properties and which also has the ability to act as a hydrogen donor for hydrogen bonding. If an acceptor is located on the enzyme in this region it would be expected that these amides would be bound more firmly than the corresponding esters. As Table I shows, this is not the case. Glutaramide VII exhibited loss of activity and succinamide VI was almost completely inactive. It is clear that no hydrogen acceptor for hydrogen bonding exists in this region of the enzyme and that, in the case of the succinamide, binding of the inhibitor actually appears to be blocked rather than unchanged or favored.

The total absence of enhanced binding by the carbon, ether, or amide analogs with respect to the ester III (m = 2) provides information about the enzyme surface in this area. Because of the lack of effect from these functional groups it is tempting to suggest that the enzyme surface contains no functional groups which aid in binding in this region.

Table II shows the $(I/S)_{0.5}$ values for alkenyl esters VIIIa-f and epoxyalkyl esters IXa-f. For comparison purposes the reference standard compound III (m = 2) is also included in the table. These data provide valuable information concerning the nature of inhibitor binding as well as considerations for future inhibitors. As a group, alkenyl esters VIIIa-f exhibited inhibition equal to reference standard III (m = 2). This could be an indication that the hydrophobic area in which the n-butyl group of III (m = 2) binds (158) is capable of accomodating the flat alkene moiety as effectively as the methylene units of the reference compound. Binding was not enhanced by the alkene function as shown by the essentially unchanged activity. Since the alkene group of esters VIIIa-f is terminal, it might be postulated that the unchanged activity could be caused by the unsaturation not participating in binding in any way. This could take place if the alkene moiety projects away from the enzyme surface when the inhibitor is bound. If this were true, it would be anticipated that the inhibition would decrease because of loss of hydrophobic binding units as compared to the n-butyl group of III (m = 2). Therefore, it is more reasonable to suggest that unsaturation in this region contributes to binding as effectively as a fully saturated alkyl group. Proposed compounds X-XII would have aided the stereochemical examination of this portion of the enzyme; however, satisfactory methods of synthesis for these

Table II: Inhibition of Rat Liver HMG CoA Reductase by



R	Compound Number	$(I/S)_{0.5}$ m = 2	Compound Number	$(I/S)_{0.5}$ m = 3
$-CH_2-CH=CH_2$	VIIIa	10	VIIIb	9
	IXa	20	IXb	13 (26% inhibition)
$-CH_2-CH_2-CH=CH_2$	VIIIc	11	VIIId	11
	IXc	13	IXd	13
$-CH_2-CH_2-CH_2-CH=CH_2$	VIIIe	11	VIIIf	8
	IXe	13	IXf	10
$-CH_2-CH_2-CH_2-CH_3$	III	11	III	11

compounds could not be developed during this investigation.

The inhibition of rat liver HMG CoA reductase by epoxyalkyl esters IXa-f is also shown in Table II. As with the corresponding alkenyl esters VIIIa-f, these compounds exhibited no general trend of either enhancing or decreasing reversible binding to the enzyme. Only in the case of the epoxybutyl system IXa and b was inhibition lowered with respect to alkenes VIIIa-f and reference inhibitor III (m = 2). This slight loss of activity appears to be unexplainable since examination of molecular models shows that the epoxide group of IX a is able to adopt conformations similar to those of the remaining epoxyalkyl esters. However, a possible explanation for the low activity of epoxy esters IXa and IXb is available. These esters are the only members of the entire epoxy ester series which are capable of forming a fairly stable ring conformation (Figure 15). In this instance the dipole interaction between the oxygen electron pair of the epoxide and the electron deficient carbonyl carbon atom of the ester provide a stabilizing influence for this conformation. It is possible that this ring stability retards esters IXa and IXb from forming the enzyme-binding open chain conformation. It should be noted that ester IXb showed 26% inhibition at its maximum solubility in the test system and therefore an $(I/S)_{0.5}$ value could not be obtained.

The most important aspect of these results is that the incorporation of the epoxide functional group into the side

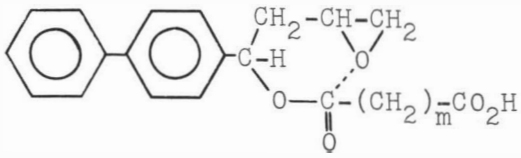


Figure 15: A possible stable conformation of esters IXa and IXb contributing to the loss of inhibitory activity.

chain definitely did not destabilize reversible binding. This is clearly demonstrated by the fact that these compounds are as active as the reference standard III (m = 2). It must be emphasized that the epoxide group is more polar than the n-butyl chain of III (m = 2) and the alkenyl chain of VIIIa-f. In addition, Boots et al. (158) have clearly demonstrated that the region in which these groups bind to the enzyme is a nonpolar area and that this binding is probably hydrophobic in nature. This strongly suggests that the epoxide oxygen of IXa-f is oriented away from the enzyme surface during binding. This conformation allows the most polar portion of the epoxide ring, the oxygen atom, to be projecting into the polar aqueous medium surrounding the enzyme while the least polar portion of the ring is free to contribute to binding on the nonpolar enzyme surface.

The present work includes only the study of the reversible binding characteristics of these compounds and provides the preliminary evidence required before irreversible binding studies may be initiated. The next phase

of this investigation will consist of prolonged preincubation of the enzyme and inhibitor followed by the addition of substrate. These experiments will provide a means of evaluating the possible irreversible inhibition of HMG CoA reductase by potential alkylators IXa-f.

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ABSTRACT OF

SYNTHESIS AND EVALUATION OF SOME ARYLALKENYL AND ARYLEPOXYALKYL HYDROGEN SUCCINATES AND HYDROGEN GLUTARATES AS INHIBITORS OF RAT LIVER β-HYDROXY-β-METHYLGLUTARYL COENZYME A REDUCTASE

BY

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Atherosclerotic disease is an almost universal phenomenon and increases in severity and frequency with increasing age. Atherosclerosis may contribute to several disorders including bursting of an artery, blockage of an artery, or induction of arterial clotting. The culmination of these diseases is usually premature since at the time of death the unaffected organs are in reasonably satisfactory condition and could have operated for several more years. Among the many factors which act in concert to produce the disease, serum cholesterol levels play a central role. It has been suggested that the lowering of cholesterol levels will provide an effective means of treatment and prophylaxis of atherosclerosis.

The purpose of this investigation was to rationally design, synthesize, and evaluate agents to lower serum

cholesterol levels by the inhibition of cholesterol biosynthesis at the site of the reduction of β-hydroxy-β-methylglutaryl coenzyme A (HMG CoA) to mevalonic acid. This reaction, mediated by HMG CoA reductase, was chosen as the inhibition target because it is the first irreversible reaction, the rate limiting step for the pathway, and the site of physiological regulation of cholesterol biosynthesis. Rat liver HMG CoA reductase provided a convenient test system for these agents.

Using the previously reported compound, 1-(4-biphenylyl)-n-pentyl hydrogen succinate as parent inhibitor, the present study accomplished two goals: first, a contribution toward elucidation of reversible binding sites for these inhibitors and second, probing of the suggested nonpolar n-pentyl binding area of the enzyme by introduction of a functional group capable of alkylating the enzyme and providing irreversible inhibition. The first objective was approached by replacing the ester of the parent inhibitor with an amide functional group. The resulting glutaramide exhibited inhibition comparable to that of the parent inhibitor. This may be taken as evidence that isosteric replacement of the parent ester group with the amide N-H did not seriously alter the ability of the inhibitor to bind to the enzyme and that an additional binding site in this region is not available. The data also support the suggestion that the ester group of the parent agent is not necessary for binding.

A similar inhibition study was made possible by synthesis of 1-(4-biphenylyl)-n-pentyl hydrogen 3-methyl-3-methoxyglutarate. With respect to the corresponding 3-methyl-3-hydroxyglutarate this compound showed an elevenfold decrease of activity. This considerable activity loss indicates that the 3-methoxy group interferes with reversible binding of inhibitor to the enzyme. The inhibition data indicate that the 3-hydroxy group of the 3-methyl-3-hydroxy compound contributes to reversible binding by participating as a hydrogen donor in hydrogen bonding with the enzyme.

The major portion of this investigation was designed to probe a region of the enzyme which is nonpolar and binds the n-alkyl moiety of the parent inhibitor. The purpose was to determine the feasibility of incorporating a functional group into this region of the inhibitor which could act as an acceptor for an enzymic nucleophile located in proximity to the reversible binding area. If successful, this could provide irreversible inhibition of the enzyme. A series of compounds was synthesized which bore a terminal alkenyl group two to four carbon atoms removed from the ester moiety. Testing showed that, with respect to the parent inhibitor, no appreciable loss of binding took place. Similarly, a series of epoxyalkyl esters was prepared, the epoxide group being the portion of the inhibitor susceptible to nucleophilic attack. Reversible binding of these compounds was found to be equal to that of the parent inhibitor

and it was therefore concluded that the enzyme does accomodate this alkylating group with no loss of reversible binding. This provided the necessary preliminary work upon which subsequent irreversible binding studies will be based.